Role of Thyroid Hormones in Pancreatic Acinar Cell Regeneration following Acute Pancreatitis

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1. Summary:

Acute pancreatitis is a debilitating inflammation of the pancreas, which leads to exocrine dysfunction; in its severe form, the disease is associated with substantial morbidity and mortality. Following pancreatitis, the pancreas exerts some regenerative capacities: in particular, differentiated acinar cells are able to re-enter the cell cycle and start to proliferate. However, the extent of pancreatic regeneration is limited; therefore, elucidating the molecular mechanisms that drive pancreatic regeneration and boost this process is a major focus of research. Thyroid hormones (TH) are important homeostatic regulators of several biological processes, including regenerative responses. Recently it was observed that TH promote pancreatic acinar cell proliferation in adult rodents, without induction of damage or inflammation.

In the present project, we sought to investigate whether TH are involved in pancreatic regeneration, and if non-physiological TH levels alter acinar cell proliferation following acute pancreatitis. Moreover, we aimed to characterize the molecular mechanisms of the mitogenic activity of T3, the biologically active TH, on adult acinar cells.

To this aim, we used the well-established model of serial cerulein injections to induce acute pancreatitis, we altered TH levels *in vivo* in both a systemic (hypothyroidism or thyrotoxicosis) and tissue specific manner in genetically modified animals and investigated how TH-related parameters in the pancreas intersect with pancreatic regeneration following inflammatory injury of the tissue.

We found that TH are locally activated at the onset of acute pancreatitis and local hyperthyroidism triggers acinar cell proliferation. Characterization of altered TH levels *in vivo* highlighted a positive correlation between TH levels and number of proliferating acinar cells, which was further demonstrated by the use of an animal model of increased pancreatic TH levels. Moreover, we observed that TH influence acinar cell proliferation independently of tissue damage or inflammation. The investigation of the molecular mechanisms of T3 induction of acinar cell proliferation revealed the involvement of Akt, HDAC and TGF-β signaling.

We concluded that activation of TH signaling is an initiating event that triggers pancreatic regenerative response, promoting acinar cell proliferation. Moreover, we observed that acute pancreatitis leads to a systemic decrease in circulating TH, which may contribute to the worsening of the disease. Overall, the present study provides the foundation for the potential use of TH to boost pancreatic exocrine regeneration and suggests reduced thyroid function may impact pancreatic regeneration, in patients suffering from acute pancreatitis. Lastly, we propose that T3 exogenous administration is an efficient model to study acinar cell proliferation *in vivo* in the absence of pancreatic damage and inflammation.

1.1 Zusammenfassung

Akute Pankreatitis ist eine schwächende Entzündung der Bauchspeicheldrüse, die zu exokriner Dysfunktion führt. In ihrer schweren Form ist die Krankheit mit erheblicher Morbidität und Mortalität verbunden. Nach einer Pankreatitis übt die Bauchspeicheldrüse einige regenerative Fähigkeiten aus. Insbesondere differenzierte Azinuszellen können wieder in den Zellzyklus eintreten und sich vermehren. Das Ausmaß der Pankreasregeneration ist jedoch begrenzt, weswegen die Aufklärung der molekularen Mechanismen, welche die Pankreasregeneration vorantreiben, ein wichtiger Forschungsschwerpunkt sind. Schilddrüsenhormone (TH) sind wichtige homöostatische Regulatoren verschiedener biologischer Prozesse, einschließlich regenerativer Reaktionen. Kürzlich wurde beobachtet, dass TH die pankreatische Azinuszellproliferation bei erwachsenen Nagetieren ohne Induktion von Schäden oder Entzündungen fördern.

Im vorliegenden Projekt untersuchten wir, ob TH an der Pankreasregeneration beteiligt ist und ob nichtphysiologische TH-Spiegel die Azinuszellproliferation nach akuter Pankreatitis verändern. Darüber hinaus wollten wir die molekularen Mechanismen von T3, der biologisch aktiven TH, mitogener Aktivität auf adulten Azinuszellen charakterisieren.

Zu diesem Zweck verwendeten wir das etablierte Modell serieller Cerulein-Injektionen, um eine akute Pankreatitis zu induzieren. Wir veränderten die TH-Spiegel *in vivo* sowohl in systemischer (Hypothyreose oder Thyreotoxikose) als auch in gewebespezifischer Weise (GMO-Tiere) und untersuchten, wie TH-bedingte Parameter in der Bauchspeicheldrüse sich mit der Pankreasregeneration nach einer entzündlichen Verletzung des Gewebes verhalten.

Wir fanden heraus, dass TH zu Beginn einer akuten Pankreatitis lokal aktiviert ist und eine lokale Hyperthyreose die azinöse Zellproliferation auslöst. Die Charakterisierung der veränderten TH-Spiegel *in vivo* zeigte eine positive Korrelation zwischen den TH-Spiegeln und der Anzahl der proliferierenden Azinuszellen, was durch die Verwendung eines Tiermodells mit erhöhten Pankreas-TH-Spiegeln demonstriert wurde. Darüber hinaus beobachteten wir, dass TH die azinöse Zellproliferation unabhängig von Gewebeschädigung oder Entzündung beeinflusst. Die Untersuchung der molekularen Mechanismen der T3-Induktion der Azinus-Zellproliferation zeigte die Beteiligung von Akt-, HDAC- und TGF-β-Signalisierung.

Wir folgern, dass die Aktivierung der TH-Signalgebung ein auslösendes Ereignis ist, welches die regenerative Pankreasresponse auslöst und die azinare Zellproliferation fördert. Darüber hinaus beobachteten wir, dass eine akute Pankreatitis zu einer systemischen Hypothyreose führt, die zur Verschlimmerung der Erkrankung beitragen kann. Insgesamt legt die vorliegende Studie Grundlagen für die mögliche Verwendung von TH zur Förderung der exokrinen Pankreasregeneration, und legt die klinische Beurteilung der Schilddrüsenfunktion bei Patienten mit akuter Pankreatitis nahe.

2. Introduction

2.1 Thyroid hormones

Thyroid hormones (TH) are important homeostatic regulators of several biological processes, which act on virtually any cell. The major product of the thyroid is thyroxine (L-3,5,3',5'-tetraiodothyronine, T4), however triiodothyronine (3,3',5-triiodo-L-thyronine, T3) is considered to be the active TH. TH play an important role during the whole life span of individuals across all vertebrates. Importance of TH during development as well as adult life is best recognized when aberrant thyroid function, thyrotoxicosis or hypothyroidism, are observed. Several conditions are associated with altered TH levels, among them cardiovascular diseases, obesity and diabetes [1]. Moreover, hypothyroidism during embryo development leads to aberrant organogenesis, particularly for the neural system [2].

As discussed in the following sections, production, transport and activity of TH are strictly regulated by multiple mechanisms that orchestrate TH control in both a systemic and a tissue specific fashion.

2.1.1 Production and release of thyroid hormones

The thyroid is the first glandular structure to form during human development and it becomes functional at approximately 10 weeks of gestation (~E15.5 in the mouse) [2]. It consists of two elongated lobules, connected by an isthmus, composed by cuboidal cells: the thyrocytes. Thyrocytes lay in spherical monolayers, the follicles, with their lumens filled with a clear protein-rich substance called colloid. Thyroid follicles are highly vascularized and surrounded by a connective capsule [3].

Synthesis of thyroid hormones:

The first step for the synthesis of TH is Iodine (I_2) uptake; Iodide (I^-) absorbed by the stomach and the intestinal tract is then transported to the thyroid, which stores about 80% of all body Iodide. Iodide is actively concentrated in the thyroid follicular cells by the action of the Iodine-Sodium-symporter (NIS); once in the cytosol, Iodide is transported in the colloid by the Pendrin transporter, which exchanges Iodide efflux with electroneutral exchangers (i.e. Cl^-). At the interface between colloid and the apical membrane of thyrocytes, the enzyme thyroperoxidase (TPO) catalyzes the organification of Iodide by promoting its incorporation in tyrosine residues of a large precursor molecule named thyroglobulin (TG - >300KDa). Specifically, the thyroperoxidase requires the action of two dual oxidases enzymes DUOX1 and DUOX2: DUOX enzymes are responsible for the production, NADPH dependent, of H_2O_2, which is necessary for thyroperoxidase to oxidize Iodide ($I^- \rightarrow I^0$). Oxidized Iodide reacts spontaneously with thyroglobulin, stored within the colloid, thereby producing monoiodinated-tyrosine (MIT) or diiodinated-tyrosine (DIT) residues. Thyroperoxidase together with H_2O_2 are responsible also for the

conjugation of two DIT residues or a MIT with a DIT thereby producing T4 and T3 residues. Thyrocytes incorporate mature TG within vesicles that are driven to the lysosome. After fusing with the lysosome, hydrolysis of thyroglobulin will generate T4 and T3 (and one alanine that is the link of TH to the backbone of thyroglobulin) which are released in the circulation. Importantly, MIT or DIT residues, as well as rT3, that are not useful for the generation of TH are reabsorbed and recycled by the activity of the iodotyrosine dehalogenase 1 (DEHAL1), which catalyzes deiodination of the iodinated tyrosine. A schematic representation of TH synthesis is presented in Fig.1 (Reviewed in [4]).

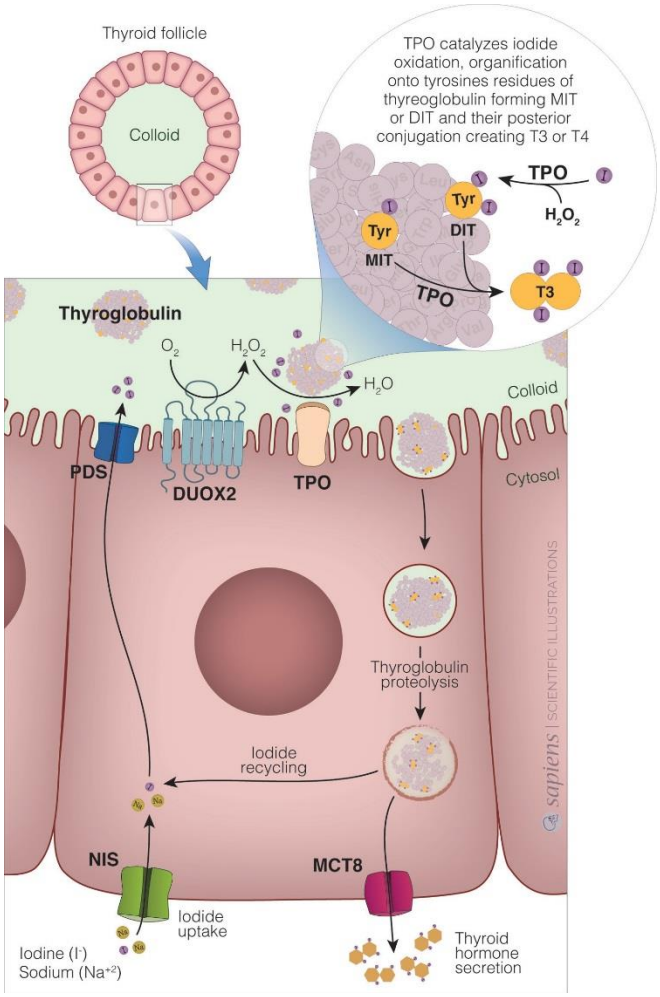


Figure 1: *Schematic representation of thyroid hormone synthesis. Taken from [4].*

Multiple signals define thyroid activity and therefore TH synthesis and release; this systemic control has been extensively studied and is referred as the hypothalamic-pituitary-thyroid axis.

Hypothalamic-pituitary-thyroid axis:

Regulation of TH synthesis in the thyroid: Thyroid function and growth is intimately regulated by the Thyroid-stimulating factor (TSH), which is produced by thyrotropic cells (also called thyrotropes) located within the pituitary gland. TSH is a glycoprotein composed of two subunits (α and β); the α-subunit is shared among different hormones released by the pituitary (i.e. vasopressin, oxytocin, GH, FSH and LH) while the β-subunit is specific for TSH [5]. TSH acts on thyroid follicular cells by binding to its own receptor (TSH-R): a G-coupled receptor able to transduce multiple signaling cascades. The main downstream signaling activated by TSH-R is the adenylate cyclase with consequent accumulation of cAMP [6]. The cAMP pathway in turn, induces transcription of key components of thyroid synthesis such as the Iodine-Sodium-symporter (NIS), thyroperoxidase (TPO) as well as TSH-R genes [5]. Furthermore, cAMP activates protein kinase A (PKA), which promotes thyrocytes growth and proliferation [7]. Recently, Selmi-Ruby et al. reported that the thyroid expresses all TH receptors, and specific removal of the TH receptor β isoform in thyrocytes dysregulates normal thyroid hormone synthesis, highlighting the existence of an additional regulation of thyroid function by direct TH feedback [8].

Regulation of thyroid-stimulating factor (TSH) in the pituitary gland: Thyrotropic cells receive signals from the hypothalamus and the circulation to modulate the release of TSH. Circulating TH, for example, can enter thyrotropic cells in which they reduce both TSH release and production [9]. Thyrotropic cells express TH receptors as well as deiodinases (DIO, enzymes able to catalyze deiodination of TH, see further); of notice, expression of deiodinase type-II (DIO2), responsible for intracellular T4→T3 activation, amplifies TH negative feedback ensuring proper synthesis and release of TSH [10]. Furthermore, TH downregulate expression of Thyrotropin-releasing hormone receptor (TRH-R) and promote its degradation reducing the stimulatory signal sent out by the hypothalamus [11]. TRH is a tripeptide (Glu-His-Pro) produced by neurons in the paraventricular nucleus of the hypothalamus, it upregulates TSH synthesis and induces fast release of TSH stored in vesicles [4]. Moreover, TRH acts as a trophic factor during thyrotropes development, promoting their growth and proliferation [12]. Importantly, aside from regulating transcription and secretion of TSH, TRH signaling promotes TSH glycosylation, which is required for proper TSH activity [13].

Regulation of Thyrotropin-releasing hormone (TRH) neurons in hypothalamic neurons: Within the paraventricular nucleus of the hypothalamus, thyrotropin-releasing hormone neurons release TRH in the pituitary-portal circulation. TRH release is controlled by several complex signals from the central nervous system, which integrates information about metabolism, body temperature, and cardiovascular function, as well as by circulating TH levels. TH are the main modulators of TRH synthesis: particularly, the TH receptor β2 (THRβ2) isoform represses TRH transcription as well as downregulates expression and activities of key enzymes involved in TRH maturation (i.e. PC1/3, [14]).

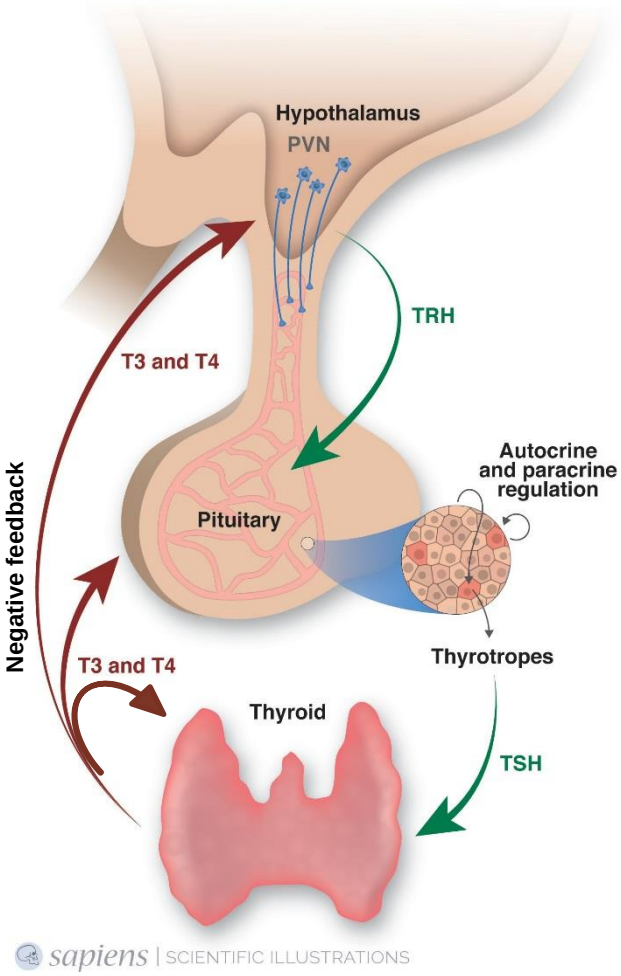


Figure 2: *Schematic representation of the hypothalamic-pituitary-thyroid (HPT) axis. Adapted by [4].*

In conclusion, the hypothalamic-pituitary-thyroid axis ensures proper synthesis and release of TH in the circulation by integrating multiple physiological signals. At the same time, circulating TH establish a negative feedback regulation with each member of the axis. Experimental models of disruption or dysregulation of many components have highlighted that, although compromised, this system "re-wire" to preserve physiological levels of TH [15].

2.1.2 Thyroid hormones transporters: within the circulation and across membranes

Because of their low solubility in water, once in the circulation T4 and T3 travel bound to proteins. The liver is the main producer of TH carrying proteins: thyroxine-binding globulin (TBG) and transthyretin (TTR, also known as pre-albumin) [1]. Furthermore, due to its high concentration in the circulation, Albumin (ALB) is also able to carry TH [16]. Most TH travel bound to TBG (~75% of T4), which shows higher affinity to TH than TTR. The amount of circulating TH-transporters is highly variable between individuals [17], especially humans, which renders the quantification of total-T4 (T-T4) and total-T3 (T-T3) hormones a valid measurement of thyroid function only when the amount of TH-transporter does not change. A more reliable measurement of thyroid function is indeed the quantification of free-T4 and free-T3 (F-T4 and F-T3), which does not account for the abundance of TH-transporters [18]. Moreover, the F-T4 and F-T3 levels reflect the percentage of TH available to enter cells; in fact, the large amount of TH bound to proteins is thought to serve as a buffer to secure availability of TH throughout the body [19]. When looking at early embryo TH levels for example, T-T4 is approximately 100 times less than physiological levels; however, measurement of F-T4 show values in the order of its biological activity. This is due to the very low abundance of circulating TH transporter during early development [20]. It is important to mention that the binding capacities of TH transporters can be modulated by several conditions such as pH or post-translational modifications (PTMs), which in turn may constitute an additional mechanism of controlling local TH availability [21].

It was originally postulated that TH could freely cross the cell membrane because of their lipophilic nature. However, pioneering studies by Christense [22] *in vitro* that highlighted the requirement of ATP for TH uptake in ovarian cancer cells and by Schwartz *in vivo* [23] that showed that TH are up-taken at different rates between tissues, suggested the presence of specific transporter mechanisms. Up to now, many TH membrane transporters have been identified, of which some of the best-characterized classes are: the organic anion polypeptides transporters (OATPs) (reviewed in [24]), the monocarboxylate transporters (MCT) (Reviewed in [25]), and the heterodimeric amino acids transporters (HATs) (Reviewed in [26]). OATPs are part of a large family of multispecific transporters, most of which are expressed in liver and kidney where they participate in processes such as detoxification [27]. Different OATPs can transport TH across the cell membrane; among them the OATP14 (OATP-F in humans) is of particular interest for its role in the brain. OATP14 shows high affinity to T4 (but not T3) and it is highly expressed in the brain vascular system where it controls the delivery of T4 across the blood-brain barrier [28]. The LAT1 and LAT2 transporters belong to the HATs family; the work of Ritchie et al [29] in *Xenopus* l. oocytes as well as in mammalian cells demonstrated that they efficiently transport TH. Interestingly, among others transporters, LAT1 and LAT2 are widely expressed across the body, which suggest that they could determine most of TH transport across the membrane [26]. Of all the MCT transporters, mainly MCT8 and MCT10 are specific TH transporters [30]. The MCT8 gene has been extensively studied because mutations in this gene are associated with mental retardation. Indeed, mice carrying MCT8 mutations show major defects in neural development, which correlate with severe brain-hypothyroidism [31]. In fact, MCT8 is required for both influx

and efflux of T3 in neurons, thereby determining the proper TH concentrations and diffusion during brain development; of note, other organs seem to be spared by MCT8 dysfunctions.

2.1.3 Intracellular regulation of thyroid hormones: the deiodinases family

Virtually any cell is able to regulate intracellular TH concentration by modulating expression and activity of TH transporters and deiodinases (DIO) enzymes. Deiodinases catalyze the mono-deiodination of TH on either the outer ring (outer ring deiodination: ORD) or the inner ring (inner ring deiodination: IRD), contributing to both activation and inactivation of TH (T4 → T3 and T4→rT3, T3 → T2 respectively – See figure 3). Three different deiodinases have been identified in both rodents and humans (DIO1-2-3). All deiodinases are integral membrane proteins that share a similar catalytic site, the thioredoxin fold, which contains the special amino acid Selenocysteine (Sec). The Sec amino acid is coded by UGA, which is usually "read" as a stop codon; however, the presence of a specific 3D loop structure, the SECIS element, present in the 3'UTR of the messenger RNA allows the insertion of this special amino acid (figure 4) [32].

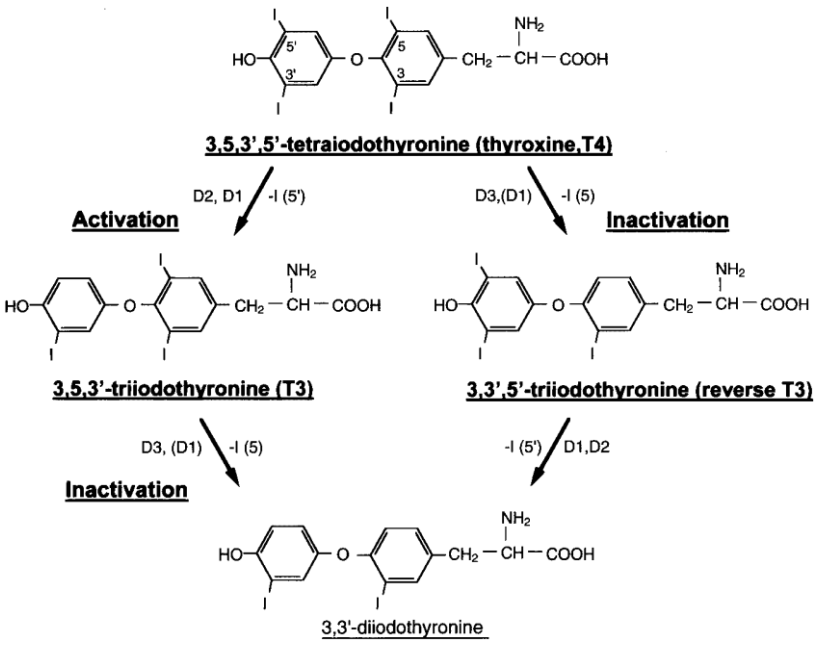


Figure 3: *Mechanism of action of deiodinases enzymes.*

(Note that here deiodinases are ascribed as D1-2-3) Taken from [32]

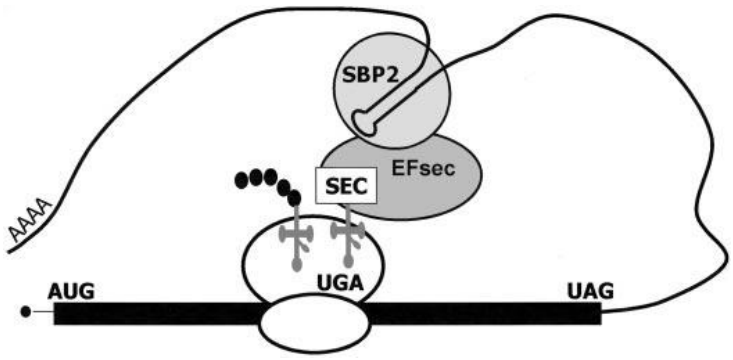


Figure 4: *Schematic representation of Selenium-Cysteine (SEC) insertion. Briefly, during translation, the SECIS element encoded in the 3' of the deiodinase mRNA, folds into a stem-loop and promotes insertion of the SEC amino acid instead of the stop-codon UGA. Taken from [32].*

Deiodinase type-I (DIO1) was the first deiodinase to be identified [33]; it is the only deiodinase able to catalyze both ORD and IRD reactions determining both activation and inactivation of TH. DIO1 is expressed in several organs such as liver, kidney, muscle and the thyroid [34]. It is well accepted that DIO1, particularly in the liver, is responsible for most peripheral T4→T3 conversion [35], however its high preference for rT3 and sulfated TH (see further: TH catabolism) suggests that it may participate in the catabolism of TH and serve as a scavenger of excess TH [32]. DIO1 expression is regulated by multiple factors, including TH themselves (a TRE element on the promoter was identified, see further). It localizes specifically on the plasma membrane with the catalytic site towards the cytosol, however retention in the ER has also been observed (i.e. in the liver [36]). Mice lacking DIO1 (DIO1KO) show elevated T4 and rT3 levels while T3 concentrations remain unchanged, arguing its importance in peripheral TH activation [34]. Interestingly, analysis of TH excretion highlighted a marked increase of TH in feces of DIO1KO animals suggesting the involvement of DIO1 in recycling iodine. **Deiodinase type-II (DIO2)** is the main intracellular activator of TH (T4 → T3); its expression increases during hypothyroidism while T4 and rT3 act as potent inhibitors of DIO2 activity [32]. Importantly, DIO2 is retained in the ER, with the catalytic site exposed to the cytoplasm: this specific localization allows the newly generated T3 to access the nucleus without being inactivated by other deiodinases. Multiple factors regulate DIO2 gene expression: among others, cAMP signaling has been described to play a major role in promoting DIO2 expression in different organs, particularly brown adipose tissue (BAT) [37, 38]. Aside from the transcriptional control, DIO2 activity is regulated at a posttranslational level by binding to T4: precisely, DIO2 shows a half-life of ~45min, and presence of T4 accelerates DIO2 degradation. Steinsapir demonstrated that this regulation depends on the proteasome activity; indeed, further studies have highlighted that substrate interaction (particularly T4) promotes DIO2 ubiquitination thereby impeding excessive T3 production [32, 39]. Animal models of DIO2 deletion (DIO2KO) show normal T3 levels but elevated T4 and TSH; as previously mentioned, this can be explained by the absence of DIO2 in pituitary cells where T4→T3 conversion amplifies TH feedback downregulating TSH production [34]. Moreover, as observed in hypothyroid animals, lack of DIO2 in BAT impairs adaptive thermogenesis ([40], see further action of

TH in metabolism). Surprisingly, no major alterations were observed with regards to cognitive functions, arguing the importance of DIO2-dependent T3 activation during brain development. **Type-III deiodinase (DIO3)** is responsible for inactivating T3 (T3→T2) and preventing T4 activation (T4→rT3); of all the three deiodinases DIO3 is the only to have an intronless and imprinted gene structure [41]. DIO3 localizes on the plasma membrane with its catalytic site directed towards the cytosol [42]; because of this specific localization DIO3 expressing cells are able to reduce TH influx and maintain a hypothyroid state independently of circulating TH levels. Baqui and colleagues have highlighted that DIO3 can be re-internalized within vesicles, in a clatrin dependent mechanism [43], while Jo and collaborators showed that DIO3 can be found in the nucleus of neurons [44], suggesting the existence of a complex regulation of DIO3 localization. DIO3 is expressed in multiple tissues (i.e. brain, skin, liver) where it attenuates TH stimulation; in the placenta, for example, DIO3 activity prevents excessive transfer of maternal TH towards the embryo. Interferences with placenta-DIO3 activity lead to abnormal embryonic development due to thyrotoxicosis [45, 46]. Interestingly, DIO3KO animals are hypothyroid but show normal TSH levels. Although still poorly understood, a possible explanation for this is that DIO3 may protect the hypothalamus from thyrotoxicosis, ensuring proper TRH production (TH inhibit TRH secretion, see previous section) [4]. Recently a great interest towards DIO3 has been evoked by multiple observations that DIO3 expression is often found in cancer cells (see further TH and cancer).

In conclusion, deiodinases ensure a fine regulation of intracellular TH. As it will be discussed in the next section, local availability of TH together with tissue specific distribution of TH receptors define a cell-specific transduction of TH signaling.

2.1.4 Thyroid hormones activity: between genomic and non-genomic actions

Within the cell, TH regulate several cellular processes; most TH signaling occurs via binding of TH with their TH receptors (THR) and consequent transcriptional regulation, a process named **genomic action** of TH. TH receptors belong to the nuclear receptor superfamily (NR) and they bind to specific DNA sequences: the Thyroid Response Elements (TRE) [47]. TH receptors share a conserved structure with other NR, consisting of a variable N-terminal domain, a DNA binding domain (DbD), a hinge region and the C-terminal domain, which allows ligand binding and receptor dimerization. Two genes encode for TH receptors in all mammals: TH receptor α (THRα) and TH receptor β (THRβ) [48]. Each gene produces different isoforms that vary for ligand-binding capacities, tissue distribution, and function (see figure 5). TH receptors act as homodimers or heterodimers by binding other nuclear receptors such as the Retinoic X receptor (RXRs). Although still poorly investigated, the specific homo- / heterodimers that are formed contribute to define the specificity and the degree of TH receptor transcriptional regulation. It follows that the relative abundance of each transcription factor constitutes an additional regulation of TH signaling [49, 50]. In contrast to classic steroid receptors, TH receptors are able to modulate transcription also in a ligand-independent manner. Particularly, un-ligated TH receptor negatively regulate transcription by recruiting corepressors (N-COR-SMRT-HDAC) while binding to TH favors the displacement of the repressor complex and recruitment of coactivators (SRT-HAT) [47]. – Represented in figure 6 - Exceptions to this model are genes negatively regulated by TH. The proposed mechanism for this regulation involves a specific DNA sequence, the “negative”-TRE (nTRE): binding of TH ligated TH receptor to nTRE generates a different TH-THR-DNA complex conformation that favors corepressors recruitment and consequent ligand dependent repression of transcription. An example of this is the regulation of the thyroid-stimulating factor (TSH-) β subunit promoter, which is negatively regulated by TH as a component of the negative feedback loop that controls the hypothalamus-pituitary-thyroid axis [51].

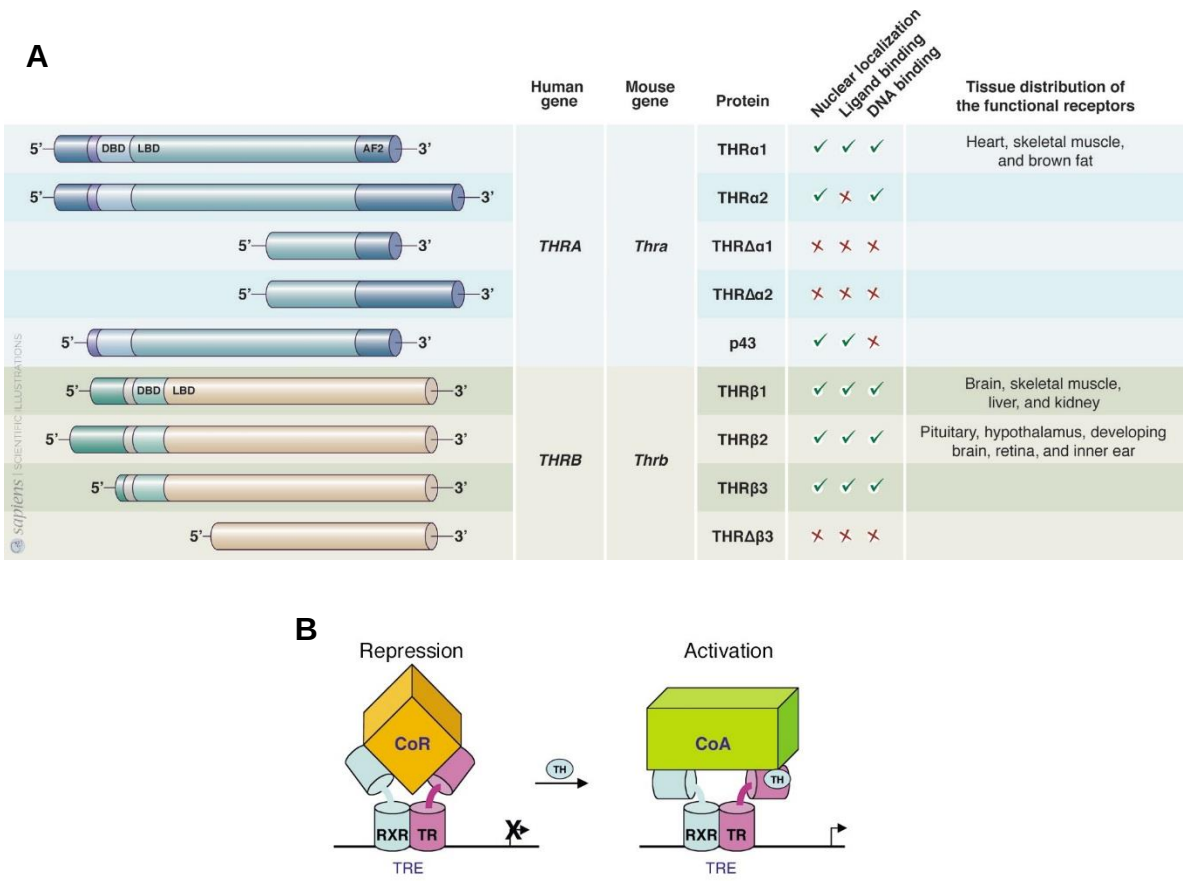


Figure 5: ***A****) Table illustrating the different isoforms identified for Human and mouse TH receptor genes (Taken from [4]).* ***B****) Respresentation of TH receptor modulation of transcription on thyroid hormone response elements (TRE). (Taken from [51])*

Although it was initially postulated that TH act solely as transcription modulators, several evidences have been collected which demonstrate that TH regulate several biological processes independently of transcription [52]. These **non-genomic actions** of thyroid hormone often involve TH receptors, furthermore they can be initiated on the cell membrane by the integrin αVβ3 (recently reviewed in [53]). The αVβ3 is a membrane receptor that mediates interaction with the extracellular matrix (ECM, i.e. laminin); it shows two binding sites for TH, one for T4 and T3 respectively; binding of TH activates multiple intracellular signaling in a ligand specific manner (T4 or T3 specific). Davis and coworkers have extensively characterized the signaling cascades transduced by αVβ3; most of these analyses used particulate analogs of TH (i.e. agarose-T4), which are excluded from the cytoplasm, allowing the selective study of signaling triggered on the plasma membrane. A schematic representation of actions initiated by the αVβ3 integrin receptor can be found in figure 6. As mentioned before, within the cytoplasm binding of thyroid hormone to TH receptors can regulate different pathways at a post-translational level. Some of the best characterized examples involve the regulation of Pi3k-Akt signaling cascade by binding

of TH receptor α or β to the p85 subunit and the crosstalk with the Wnt signaling by interaction of TH receptors with β-catenin (Reviewed [54]). It is worth mentioning that TH regulation of these pathways often transduces different cellular processes in a cell specific manner. In summary, as shown in figure 6, TH act on several cellular processes defining a complex regulation of cellular behavior.

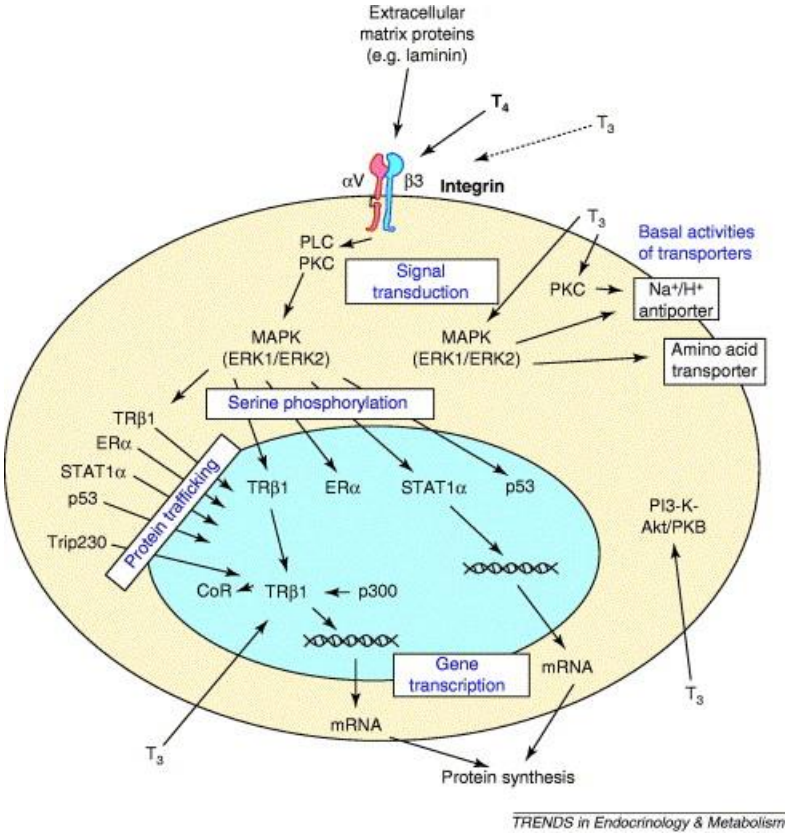


Figure 6*: Schematic representation of nongenomic actions of thyroid hormones. To note, in the present representation particular focus is given to TH actions transduced trough the integrin αVβ3. Taken from [55].*

2.1.5 Metabolism of thyroid hormones

Aside from the deiodination of TH catalyzed by deiodinases enzymes, TH are subjected to different modifications in order to modulate their catabolism and activity. Two main modifications have been described in both humans and rodents: sulfation and glucoronidation (Reviewed in [56]). Both sulfation and glucoronidation of TH are reversible modifications that increase solubility and direct TH for degradation and excretion; importantly, sulfated-TH as well as glucoronidated-TH do not show binding capacities to TH receptors and are therefore considered biologically inactive [57]. Sulfotransferase enzymes are mainly expressed in the liver. Among them they show a conserved preference for TH substrates (T2>T3≈rT3>T4) [57]. Sulfated-TH are the primary substrates for DIO1 activity, which catalyzes their deiodination, contributing to iodine recycling [58, 59]. Glucoronidation is a common modification observed to increase solubility of several compounds, in the liver glucoronidation of TH drives glucoronidated-TH for biliary- fecal excretion. It is important to mention that hydrolysis of sulfated- or glucoronidated-TH within the intestine may serve as a pool for reabsorption of active TH. Although deiodination, sulfation, and glucoronidation are the main TH modifications, also decarboxylation or deamination of the alanine side chain have been observed [56]. Decarboxylation of TH produces iodothyronamines, while deamination leads to the acetic acid TH analogues (i.e. TRIAC and TETRAC). These compounds show thyromimetics activities, however still little is known about their biological importance. Of note, the interest towards this class of compounds is rising over the last years because of their potential clinical use. TRIAC for example, show high binding specificity towards TH receptor β1 isoform and is able to cross the brain blood barrier in a MCT8 independent way, for this reason it is currently under evaluation for the treatment of subjects with MCT8 mutations [1].

2.1.6 Thyroid hormones' actions

As described in the previous sections, an intricate regulation of TH transporters, receptors, deiodinases, and other factors orchestrate TH signaling in a cell specific manner. This in turn, may explain the pleiotropic effects driven by TH during development as well as adult life. Even before completing thyroid development, the embryo actively responds to maternal TH, which can be detected as early as E11.5 – E12.5 in the mouse ([60], G5.6W in humans [61]). Proper maternal TH is indispensable for a variety of developmental processes, particularly neurogenesis [2]. Clinical conditions of maternal hypothyroidism during pregnancy are associated with mental retardation and cretinism [20]. In the following sections, examples of TH action will be discussed. In particular, the topics chosen as examples cover TH involvement in regenerative and homeostatic signaling, metabolism, and cancer.

2.1.6.1 Thyroid hormones and regulation of metabolism

TH are important modulators of metabolism, their pleiotropic actions on key metabolic organs, such as liver or muscles, as well as modulation of the nervous system regulate body energy expenditure and thermogenesis. In this section, examples of TH action on metabolism will be discussed briefly, however it is important to mention that many actions of TH remain elusive and further studies are needed to characterize their intricate regulatory network. TH increase body energy expenditure and oxygen consumption, one reason for this effect is the stimulation of Ion gradients across membranes and consequent increase of ATP usage [40]. Indeed, TH stimulate activities of the Na+/K+/ATPase on the plasma membrane [62] and the Ca^{2+} gradient between cytoplasm and sarcoplasmic reticulum within the skeletal muscle [63, 64]. Moreover, TH are important regulators of adaptive thermogenesis, this is highlighted by the observation that hypothyroid individuals are cold intolerant while hyperthyroid ones do not tolerate heat. Their regulation of adaptive thermogenesis mainly involves actions on the brown adipose tissue (BAT) and the skeletal muscle. BAT is the main tissue responsible for adaptive thermogenesis in rodents (and newborns humans), its development and function is dependent on the synergistic activities of TH and the sympathetic nervous system (SNS). Within the BAT, heat is generated by uncoupling ion gradients from the oxidative phosphorylation and consequent release of energy as heat; this proton leakage occurs on the inner mitochondrial membrane in the presence of Uncoupling protein 1 (UCP1). TH regulate expression levels of UCP1 in brown adipocytes in a TH receptor β dependent manner, however studies using selective TH receptor β agonist revealed that this TH receptor β-dependent action is not sufficient to sustain adaptive thermogenesis [65]. Indeed, TH receptor α-KO animals have impaired thermogenesis, which suggests that also the isoform α participates in this process, probably by supporting SNS stimulation of the BAT (Calcium gradient) [66]. Importantly, intracellular activation of TH via deiodinase type-II (DIO2) activity is required to generate proper TH response within brown adipocytes; DIO2KO animals are in fact cold intolerant and only T3 replacement (not-T4) stimulates BAT activity [67]. Recently, a great interest towards TH action on the adipose tissue has been generated by the observation that white adipocytes can be converted to a more brown phenotype, “brite” adipocytes, characterized by expression of UCP1 and multilocular fat storage [68]. Although still poorly understood, by regulating BAT specification and function [69], TH may serve as potent stimulators of this brite adipocytes conversion [70]. Aside from regulating adaptive thermogenesis, TH can modulate nutrient metabolism. For example, TH regulate maturation and activity of pancreatic β-cells [71-74], as well as glucose transporter (GLUT4) in muscle and adipose tissue thereby contributing to glucose homeostasis [75]. Furthermore, TH regulate hepatic fatty acids and cholesterol synthesis and catabolism (Reviewed in [76]). Overall TH levels regulate multiple metabolic processes in different tissues; importantly, the CNS in turn, integrates several signals of the “metabolic state” and regulates TH synthesis and release accordingly.

2.1.6.2 Thyroid hormones in homeostatic and regenerative processes

Participation of TH in orchestrating development of multiple organs has been observed for a long time; recently, an increased amount of evidences highlighted their continuous involvement in adult homeostasis as well as regeneration of many target tissues.

The **skin** is an epithelial organ which shows high cellular turnover, TH promote epidermal differentiation and keratinocytes proliferation during fetal development [77]. Moreover, hypothyroidism leads to the manifestation of myxedema, characterized by aberrant deposition of glycosaminoglycan and formation of dermal edema. Effect of thyrotoxicosis on skin homeostasis is, on the other hand, still controversial. Safer and coworkers described the positive effect of TH on keratinocytes proliferation *in vitro*, moreover they highlighted the importance of local TH control by deiodinases within these cells, both *in vivo* and *in vitro* [78, 79]. These data are in agreement with what has been observed in mice lacking TH receptor α and β (or both), where keratinocytes proliferation was reduced in a similar manner as observed in hypothyroid animals [80]. However, depending on the route of administration, divergent results were obtained when TH effect was tested *in vivo* [81]. While topical application of T3 induced keratinocytes proliferation, confirming the positive effect, systemic injections of T3 (injected intraperitoneal) reduced dermal thickening and proliferation. The authors proposed that systemic administration might result in release of anti-proliferative factors from other cell sources, such as dermal fibroblast, or that compensatory activity of inactivating deiodinases would inhibit T3 action on keratinocytes. Analogous results were observed when studying the effect of TH during wound healing: hypothyroid mice showed reduced proliferation and repair while administration of T3 promoted wound healing. However, analysis of positive regenerative markers, found up-regulated when T3 was administered, identified only negative TRE response elements, pointing out a more complex regulation and suggesting that T3 action might be indirect [82].

The **intestine**, as the skin, is an epithelial tissue characterized by high turnover. The involvement of TH in intestine development as well as postnatal remodeling (maturation) has been extensively studied in mice and amphibians [83]. Interestingly, the intestine has been of particular interest during the last decades because of its structure, which offers an ideal model to study stem cell function [84] and cancer progression [85]. A mature intestine consists of a pluristratified epithelium organized in villus and intervillus spaces (Crypts); within the crypts a pool of resident stem cells slowly divide and give rise to proliferating progenitors that in turn differentiate while moving towards the villus. Signaling responsible for maintaining adult stem cells and driving their progeny maturation and differentiation have been studied in detail, and the Wnt/β-catenin pathway has emerged as one of the main regulators of this process. Recently, the work of Plateroti and coworkers has demonstrated that TH participate in this process, establishing an intimate crosstalk with the Wnt/β-catenin pathway (reviewed in [83]). Particularly, the authors highlighted the importance of liganded TH receptor α in supporting Wnt activation and promoting proliferation of the intestinal epithelium [50]; furthermore, they observed the direct interaction of TH

receptor α/β-catenin/TCF complex and the negative action of THRΔα (Which acts as a negative TH receptor as it has no ligand binding capacity).

This positive effect of TH in promoting cell proliferation can be extended to others tissues such as liver, pancreas and kidneys [86]. With regards to the **liver,** the first observations that the thyroid function correlates with liver regeneration are dated back to the 1930s [87]; since then many laboratories have analyzed the relation between TH and liver regeneration [88-95]. From these studies, two main conclusions can be draw: physiological levels of TH are required for proper liver regeneration and T3 acts as a potent mitogen on liver hepatocytes. More recently, the work of Columbano and coworkers characterized the molecular mechanisms of these TH actions. They reported that T3 induces hepatocytes proliferation in a TH receptor β dependent mechanism [96], by promoting β-catenin stabilization and activity [97] with consequent activation of cyclin D (a known marker of hepatocytes proliferation). Aside from their participation in liver regeneration, TH regulate hepatocytes function and metabolism.

Although the presented examples indicate a conserved positive regulation of cellular proliferation by TH, other organs modulate TH activity for other purposes. **Skeletal muscle** is an important target of TH activity, which regulate function, homeostasis and metabolism (recently reviewed in [98]). Vice versa, the skeletal muscle regulates peripheral TH conversion, thereby modulating TH systemic levels. Indeed, deiodinase type-II (DIO2) conversion within myoblasts accounts for roughly 40% of total T3 peripheral activation and their contribution increases in hypothyroid individuals. During postnatal development, the rise in TH levels promotes the maturation of muscle fibers and their increase in size (mainly hypertrophy). In particular, TH regulate via their TH receptors (mainly the α isoform) expression levels of MYOD1, the master regulator of myoblast differentiation. Hypothyroidism is associated with incomplete muscle differentiation and improper maturation of muscle fibers [99]. In addition to the regulation of skeletal muscle function and metabolism, recent findings pointed out the importance of TH during muscle regeneration. The muscle shows some regenerative capacities: repopulation of damaged fibers relies on the satellite cells, which are quiescent adult progenitors intercalated within the myoblast. Following loss of myofibers, satellite cells re-enter the cell cycle and proliferate; consequently proliferating progenitors exit the cell cycle and differentiate in novel myofibers. As observed in postnatal muscle maturation, TH activity promotes differentiation of satellite cells; characterization of muscle regeneration in satellite cells lacking deiodinase type-II or type-III (Induced by PAX8-CRE) activities pointed out a crucial role of deiodinases in regulating intracellular TH availability during this process. In particular, following damage satellite cells upregulate deiodinase type-III expression reducing intracellular TH activation, this hypothyroid state allows the cells to enter the cell cycle and amplify the progenitor pool [100]. Following a wave of cell divisions, proliferating progenitors downregulate deiodinase type-III and activate deiodinase type-II, switching from a hypothyroid to a hyperthyroid state; this in turn promotes MYOD1 expression and consequent differentiation of progenitor cells into mature myofibers [101].

In conclusion, TH participate in multiple homeostatic/regenerative processes establishing different networks in an organ and cell specific manner. Importantly, TH are able to either enhance or block proliferation according to the target tissue; thereby suggesting that this conserved system of "TH responsiveness" (TH related factors: receptors, deiodinases, transporters) transduces different signals based on the relative amounts of the partners it interacts with.

2.1.6.3 Thyroid hormones and cancer

Considering their pleiotropic actions observed in various tissues of the body, is not surprising that TH activity is also associated with cancer formation and progression (Recently reviewed in [102]). Indeed, many types of cancer show dysregulated TH signaling or driving mutations in TH related genes. However, it is important to mention that until now many divergent effects of TH have been described according to the tumor and the model of investigation.

The first evidence that deregulation of TH signaling is involved in cancer formation came from the observation that the proto-oncogene c-ErbA other is not that a mutated TH receptor α [103]. TH receptor α plays an important role in intestinal development and homeostasis, by interacting with the Wnt signaling ([50] See previous paragraph). Kress and collaborators reported that overexpression of TH receptor α in intestinal cells is able to trigger hyper-proliferation of the intestinal epithelium and consequent adenoma formation; however the authors observed that over expression of TH receptor α *per se* is not able to induce cancer formation but acts as a tumor promoting factor when mutated APC is present [104]. The TH receptor isoform β is aberrantly expressed in different cancers, most often mutations lead to gene silencing or disruption of the TH binding capacities [105, 106]. Multiple studies on cancer cell lines revealed that re-expression of wild-type receptor can antagonize oncogenic signaling such as Kras, Pi3k-Akt, or Wnt and reduce proliferation and survival of cancer cells [107, 108]. This has been observed also *in vivo*, in a model of hepatocellular carcinoma (HCC), in which TH promote tumor regression while hypothyroidism favors tumor initiation [109]. Particularly, the authors observed that the beneficial effects of TH were abolished when TH receptor β was silenced. Later, the same group demonstrated the efficacy of a selective TH receptor β ligand (GC-01) to induce tumor regression as previously reported using T3; importantly the authors highlighted that GC-01 was not as efficient as T3, suggesting the putative involvement of TH receptor α isoform [110]. In contrast, results obtained in Insulinoma cells, demonstrated that TH receptor β acts as a pro-survival signal in these cells by mediating Akt activation in a T3-dependent fashion [111]. Furuya and coworkers analyzed the effect of a genetic mutation in the TH receptor β isoform observed in a patient with TH-resistance [112]. Particularly, they generated a mouse model carrying a mutated TH receptor β (named THRβ-PV), which has completely lost binding capacities to TH and therefore acts as a dominant-negative of TH genomic action. THRβ-PV mice spontaneously developed follicular thyroid tumors characterized by constitutive activation of Akt, a feature also observed in humans. The authors demonstrated that mutated TH

receptor β was responsible for the over-activation of Akt; indeed, THRβ-PV has increased binding capacities to p85α and leads to aberrant activation of the Pi3k-Akt signaling cascade. Despite the involvement of TH receptors α and β, a great interest towards the role of the integrin αVβ3 receptor in tumorigenesis has recently rose. This is due in particular to the observation that integrin αVβ3 is highly expressed in dividing endothelial cells and many tumor cells. Recently reviewed in [113], the group of Davis propose that TH, through the integrin αVβ3, can act as promoters of proliferation and survival on different cancer cells; furthermore, they highlight the major contribution of T4, rather than T3, in modulating these actions.

Deiodinase type-III is referred to as an oncofetal protein: its expression is often increased in solid tumors and is associated with high proliferation (reviewed in [114]). An example of this concept can be found in the basal cell carcinoma skin tumor where TH treatment inhibits tumor progression and metastasis formation [115]. Furthermore, with regards to colorectal cancer, Dentice and collaborators demonstrated that Deiodinase type-III is highly expressed in cancer stem cells, which are characterized by high activation of the Wnt/β-catenin signaling [116]. Particularly, the authors reported that β-catenin promotes upregulation of Deiodinase type-III, thereby preventing local increase of TH. The same group observed that T3 treatment can sensitize cancer stem cells to chemotherapeutics by counteracting the Wnt/β-catenin signaling and promoting a more differentiated phenotype [117]. Aside from the Wnt/β-catenin other signaling involved in cancer have been reported to promote DIO3 (Reviewed in [45]).

Furthermore, TH metabolic actions have been shown to affect tumor cells growth and survival. Many cancer cells generate energy through aerobic glycolysis, this shift in metabolism has been ascribed as “the Warburg effect” and appears to confer transformed cells growth and survival advantages (Reviewed in [118]). TH, by stimulating mitochondria biogenesis and function (see TH and metabolism), can antagonize the Warburg effect in breast cancer cells, resulting in chemotherapeutic sensitization [119].

The above-mentioned observations are only a few examples of the many evidences collected with regards to the actions of TH in cancer. As described in developmental and homeostatic processes it appears clear that TH affect tumor formation and progression in a context dependent manner, which possibly rely on the specific driving tumorigenic signaling.

2.2 The pancreas

The pancreas is a multifunctional organ, which can be divided into two main compartments: the exocrine and the endocrine pancreas. The exocrine pancreas consists of acinar cells that produce digestive enzymes and the ductal tree, which brings these digestive enzymes to the duodenum and secretes bicarbonate that reduces stomach acidity. Acinar cells are protein "factories", specialized in synthesis and secretion of digestive enzymes; they are characterized by a high number of mitochondria, an extensive endoplasmic reticulum, and electron dense vesicles filled with digestive enzymes, named Zymogens. Multiple neurohormonal regulators, named secretagogues, regulate synthesis and release of zymogens (i.e. Cholecystokinin, acetylcholine etc. [120]). On the other hand, the endocrine pancreas regulates glucose homeostasis, by releasing hormones in the circulation. The endocrine pancreas is composed of the islets of Langerhans, interspersed clusters of cells that produces key metabolic hormones such as Insulin, Glucagon and Somatostatin. (See representation in figure 7).

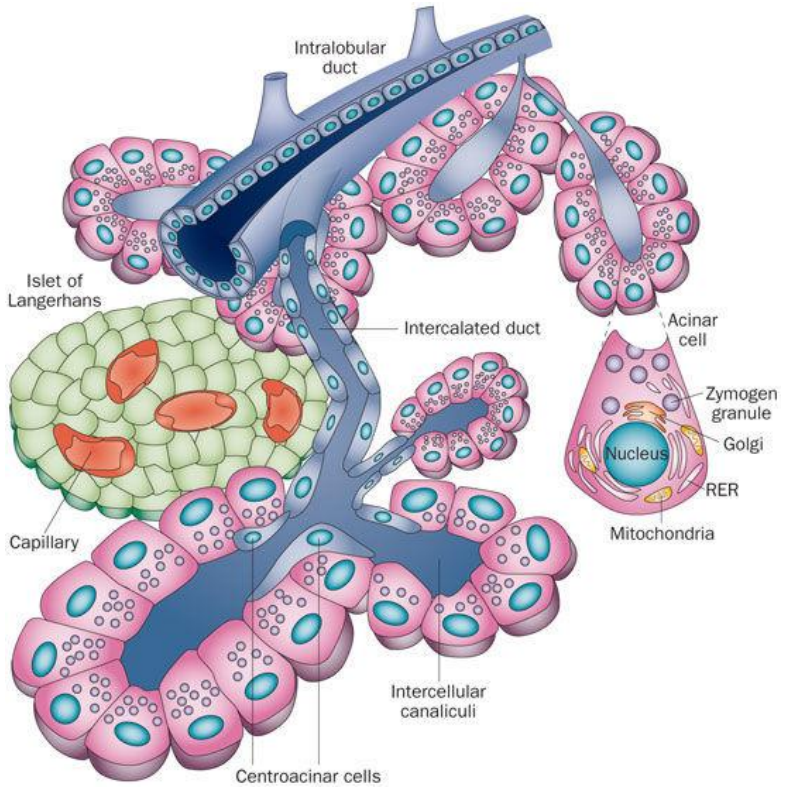
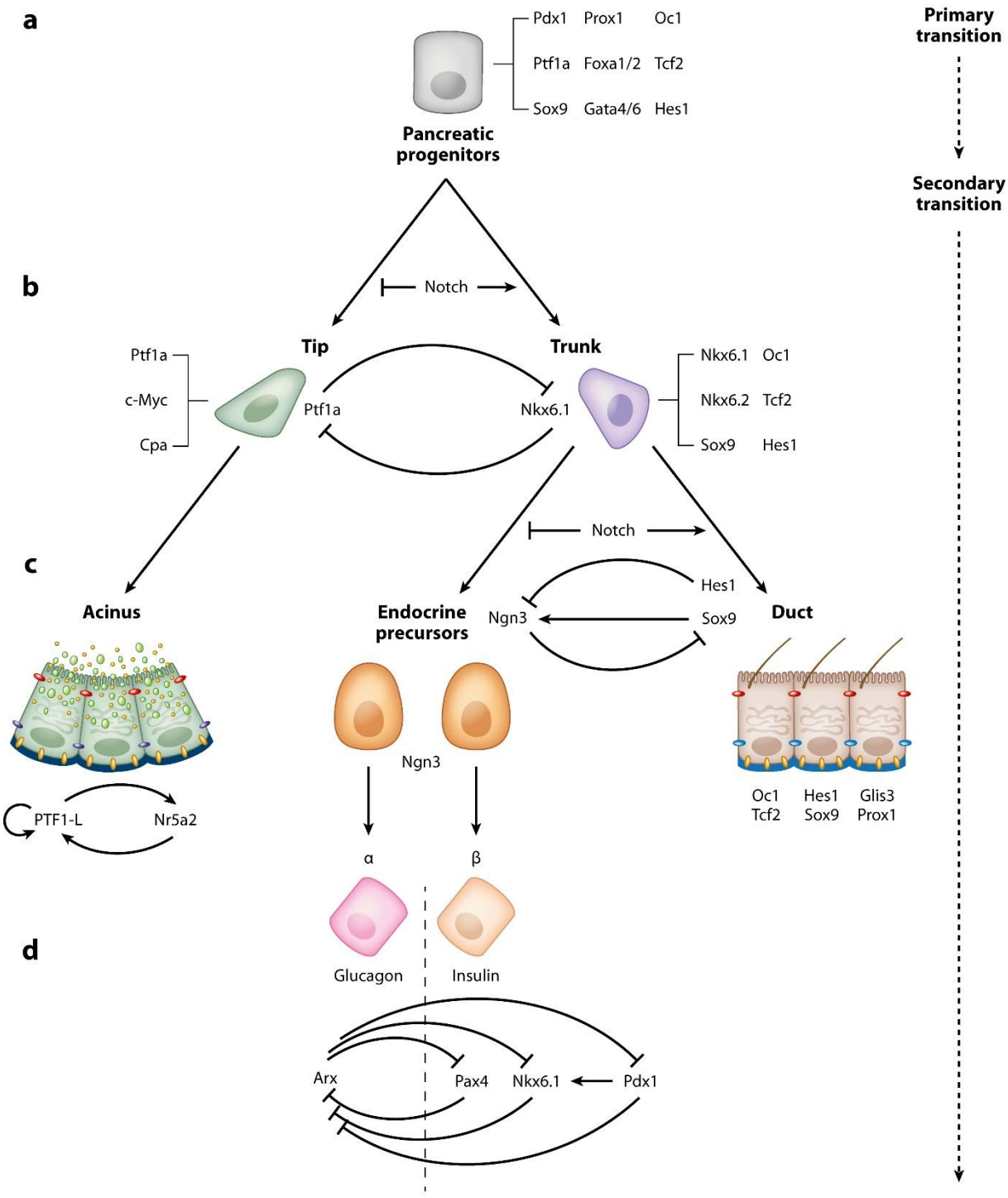


Figure 7*: Representation of the cytological architecture of the pancreas. Taken from [121].*

2.2.1 Pancreas development

In the last two decades a great effort in elucidating the molecular signaling that drive pancreatic development has brought a considerable amount of knowledge on this process (recently reviewed in [122]). In mammals, the pancreas originates from two distinct primordial buds (ventral and dorsal) that harbor independently from the foregut endoderm (Embryonic day 9 - E9 – for the mouse / gestation week 3 - G3W – in the human). Both pancreatic buds are composed of multipotent progenitor cells (MPC) expressing the pancreatic and duodenal homeobox 1 (Pdx1); the ventral and dorsal pancreas eventually fuse in a single organ and elongate (E11.5-12.5 / G5.6W). These early stages of pancreas development, referred to as **primary transition,** involve multiple determinant factors released particularly by the adjacent cardiac mesoderm. The **secondary transition** starts with expansion of the pancreatic epithelium, followed by differentiation of the MPC into adult pancreatic cells and tissue remodeling (From E12.5 until birth / ~G8W). While many signaling that drive pancreatic lineage specification have been elucidated, still little is known about pancreatic morphogenesis [123]. Notch signaling is one of the first restrictors of MPC competence: it specifies acinar precursors (Notch negative) and bi-competent ductal-endocrine cells (Notch positive); a process referred to as Tip-trunk segregation. Subsequently, the "mutually-exclusive" Ptf1a/Nkx6 switcher (two important transcription factors for pancreatic lineage specification) further consolidates Notch bimodal discrimination: on-Notch promotes Nkx6 expression, which in turn block Ptf1a, promoting an endocrine or duct fate; conversely off-Notch increases Ptf1a expression and represses Nkx6 [124]. A schematic representation of pancreatic lineage signaling is presented in figure 8.

Together with the differentiation of pancreatic progenitor cells, their proliferation ensures that the pancreas reaches the proper mass at birth. Most of our knowledge on the signaling involved comes from animals models with selected genetic alterations. The first signal known to promote proliferation of pancreatic progenitor cells is FGF (Fgf10), released from the adjacent mesenchyme [125]. More so, Wnt/β-catenin [126], Notch and Indian hedgehog (IHH) [122] among others have been described to be essential for pancreatic growth. It is worth mentioning that precise timing of regulation is crucial for proper pancreatic development: an example of this concept is well described by the work of Heiser and colleagues [127]. The authors characterized the effect of constitutive β-catenin activation (canonical Wnt effector) using two PDX1-driven recombinase Cre systems (named CRE^{early} and CRE^{late}) differing for about one day of expression (E10.5 vs E11.5). Comparison of the two mouse strains revealed that early activation of β-catenin induces pancreatic hypoplasia and prevents differentiation of MPC while the late induction results in normal differentiation and increased pancreatic growth. Overall an intricate network of signaling orchestrate pancreatic development in a spatial and time dependent fashion; as will be discussed in later sections the same key factors are often reactivated following pancreatic injury and participate in the regeneration of this organ.



Shih HP, et al. 2013.
Annu. Rev. Cell Dev. Biol. 29:81–105

Figure 8*: Schematic representation of the key factors involved in pancreatic lineage specification. Taken from [128].*

2.2.2 Pancreatic diseases

Although the most studied pancreatic diseases involve the endocrine pancreas (i.e. diabetes), the exocrine compartment also suffers many illnesses. Pancreatic exocrine inflammatory injuries, referred to as pancreatitis, can be divided into **acute** and **chronic** based on the time of persistence of the injury. Acute pancreatitis is the leading cause of hospitalization amongst gastrointestinal disorders in the United States; its severity varies from mild cases that spontaneously resolve themselves to severe pancreatitis, which leads to systemic inflammation and often mortality due to multiorgan dysfunction [129]. Chronic pancreatitis on the other hand, is characterized by the persistence of damage and inflammation within the pancreas for a long period, which often leads to acinar cell atrophy and, as previously mentioned, to pre-cancerous lesion formation [130]. Furthermore, chronic pancreatitis leads to a high fibrotic response by proliferation of resident pancreatic mesenchymal cells, often referred to as pancreatic stellate cells (PSC) [131]. Many risk factors have been associated with the incidence of acute and chronic pancreatitis: among them gallstones formation with physical obstruction of the common pancreatic-bile duct, smoking, and alcohol consumption. Most of our knowledge on the initiation and the progression of the disease comes from animal studies. The initiating event in pancreatitis is the premature activation of digestive enzymes within the pancreatic tissue and consequent self-digestion of the organ [132]. This process can be mimicked in the mouse by administrating supra-physiological doses of cerulein, an analog of cholecystokinin (CCK), which recapitulates this aberrant release and activation of digestive enzymes [132]. Following cerulein treatment inducing the initial cell damage, injured acinar cells release several cytokines that attract inflammatory cells within the pancreas. Concomitantly with inflammation, the pancreas triggers a regenerative response by which differentiated acinar cells re-enter the cell cycle and start to proliferate [133]. Moreover, injured acinar cells can transiently dedifferentiate; in this instance, acinar cells lose their zymogens content and acquire a ductal phenotype by re-expressing progenitor markers, a process known as acinar-to-ductal metaplasia (ADM) ([134, 135]). ADM typically resolves itself over time; however, persistent (chronic) pancreatitis can lead to sustained ADM formation and subsequently to pre-cancerous lesions.

2.2.3 Pancreatic exocrine regeneration

Tissue regeneration follows different mechanisms accordingly to the organ and the severity of injury investigated. While, for example, muscle and intestine show resident stem cells [136] able to repopulate the tissue following damage, the liver regenerates mainly through proliferation of differentiated hepatocytes [137]. Several studies have tried to identify resident stem cells in the adult pancreas; however, the lack of specific markers together with the plasticity observed by differentiated cells during injury have led to inconclusive results. One of the most "speculated" putative stem cell population are the centroacinar cells (CAC), which in Zebrafish have been demonstrated to serve as progenitors for both endocrine and exocrine cells [138-140]. Although a precise characterization of CAC progenitor properties in mammals is still missing, Furuyama and coworkers observed that $Sox9^{+}$ duct cells (included CAC) sustain pancreatic tissue homeostasis and are able to differentiate in acinar cells [141, 142]. However, using a similar lineage tracing system, Kopp reported that $Sox9^{+}$ cells act as progenitors only during embryogenesis and do not participate in adult tissue homeostasis or regeneration [143]. Recently two different studies, using lineage-tracing labeling of acinar cells, demonstrated the existence of high heterogeneity within the acinar compartment. Wollny et al. [144] used a low-labelling multicolor approach to track proliferation of small cluster of acinar cells over time; their analysis identified a subpopulation of proliferating acinar cells ($Stmn1^{+}$) which appear to sustain alone the homeostatic tissue turnover. Furthermore, they demonstrated that upon injury non-proliferating acinar cells ($Stmn1^{-}$) are able to express Stmn1 and proliferate to compensate for tissue loss. Westphalen and colleagues [145], on the other hand, identified a subpopulation of quiescent acinar cells ($Dclk1^{+}$) which acts as facultative progenitor and demonstrated that $Dclk1^{+}$ cells are required for pancreatic regeneration following pancreatitis (progenitor hypothesis). Whether these two subpopulations overlap has not yet been investigated, however both studies highlight that exocrine regeneration does not depend on resident stem cells. It is important to mention that exceptions to this observation have been described in models of severe acinar cells injuries (acinar cell ablation); in this scenario in fact, ductal cells can serve as progenitors and are able to repopulate the acinar compartment [146], a feature observed also in liver regeneration [147]. Although the contribution of other cell types to exocrine regeneration is still under debate, proliferation of differentiated acinar cells appears to sustain most pancreatic recovery. During the last decades, a great understanding of the signaling required for proper exocrine regeneration has been generated, among others the IGF-I [148], Wnt/β-catenin [149], HDAC [150] and TGF-β [151] have been demonstrated to regulate acinar cell proliferation during exocrine regeneration.

Interestingly, comparing regenerative and developmental signaling shows a high degree of similarities. Examples of this include the re-expression of PDX1 within the acinar compartment following pancreatitis, the increased Notch expression in transiently de-differentiated acinar cells (ADM), or the Wnt-β-catenin pathway, already described in pancreatic development. As an example, starting from the pancreatic secondary transition (~E12.5) β-catenin (canonical-Wnt signaling) is required for proper expansion of committed acinar cells; Keefe and coworkers investigated if this requirement is retained during postnatal life and pancreatic regeneration. They

observed that β-catenin in indispensable for postnatal growth of acinar cells, furthermore induction of pancreatitis to mice harboring pancreas specific deletion of β-catenin resulted in a marked decrease in acinar cell proliferation, while no differences in the level of pancreatic damage or inflammation were observed [149].

In conclusion, much of the signaling that drive pancreatic organogenesis reawake during tissue regeneration [152]; importantly, many of those pathways do not necessary resemble their developmental function and may triggers different signals. Interestingly, a similar reawakening of developmental signaling has been observed also with regards to the development of pancreatic cancer (recently reviewed in [153]).

2.3 Thyroid hormones and Pancreas

TH affect both the development as well as the adult stage of the pancreas. Much of our knowledge about the action of TH during pancreas development comes from studies of amphibian metamorphosis. Amphibian pancreas development shares many similarities with mammalian's one but differs particularly on the morphogenesis of the adult pancreas. The anuran (tadpole) pancreas consist of a "spongy" tissue which lacks any ductal system; at the onset of metamorphosis the pancreas undergoes dramatic changes that involve mainly cell dedifferentiation and apoptosis (also referred as "partial degeneration followed by regeneration" – Janes, 1937 [154]). This process is entirely TH dependent, in fact TH appear to be essential to inducing the dedifferentiation of pancreatic cells but seems disposable for the re-differentiation phase [155, 156]. Similarly to the amphibian counterpart, mouse pancreatic explants (E12.5) treated with T3 show acinar dedifferentiation and increased expression of ductal marker Sox9 [157], a process also observed in adult primary acinar cells when TH receptor α (THRα) is overexpressed and T3 added to the culture media [158]. Interestingly, the authors [157] demonstrated the pancreatic expression of TH receptor α as early as E12.5, which coincides with the beginning of the secondary transition when cell lineage specification begins. It is worth mentioning that this developmental stage corresponds to the earliest detection of maternal TH within the embryo of both rodents and human [61] as well as the earliest activation of TH signaling documented during murine embryogenesis [60]. A possible mechanism for this TH dependent dedifferentiation of acinar cells may involve the Notch signaling, which in *Xenopus l.* is directly activated by TH [159]. In fact, forced activation of Notch during pancreas development promotes the differentiation of pancreatic progenitors towards a ductal phenotype at the expenses of acinar and endocrine lineages, similar to that reported for TH [160, 161]. Studies in later stages of development or postnatal life highlighted different functions of TH in pancreas organogenesis. Harris and colleagues for example, reported that perinatal hypothyroidism increases β-cell proliferation and insulin production during sheep development, without affecting pancreatic size [162]. This is in strike contrast to what was observed in the rat, in which T3 appears to promote β-cell maturation and increases pancreatic growth, mainly due to increased number of acinar cells, during early post-natal life [163, 164]. These discrepancies observed with regards to TH actions on the pancreas may be explained by interspecies differences, or by changes in the responsiveness properties of

pancreatic cells during development, which involve differential expression of TH receptors as well as deiodinases enzymes. Analyses of TH receptors expression within the developing pancreas revealed a shift from almost only TH receptor α expression, at early stages (E12.5) to relative prevalence of the TH receptor β isoform at birth [157], these changes correlate with a different cellular localization of TH receptor isoforms α and β in early postnatal life [164]. In support of this hypothesis, analysis of the effect of T3 supplementation in adult pancreas revealed no induction of acinar cell dedifferentiation, as observed during development; on the contrary, several studies highlighted that TH promote acinar cell proliferation in a TH receptor β dependent mechanism [86, 96, 165]. Overall, multiple evidences of an intimate relationship between TH and pancreatic development and function exist, however further analyses on the expression and distribution of TH receptors, deiodinases as well as the integrin αVβ3, are required to better clarify the role of TH within the pancreas.

3. Aim of the project

Following acute pancreatitis, pancreatic acinar cells are able to re-enter the cell cycle and start to proliferate. However, pancreatic regenerative capacities are limited, and persistence of damage often leads to pancreatic insufficiency. Although many of the signaling that drive acinar cell proliferation have been characterized in the last two decades, little is known about the initiating events that trigger this process. Based on the observations that TH participate in pancreas development and act as mitogens on adult pancreatic acinar cells, their putative involvement in pancreatic regeneration seems plausible. In the present project, we sought to characterize the involvement of TH during pancreatic acinar cell regeneration and the molecular mechanisms of T3-driven acinar cell proliferation.

In particular, the following questions were addressed:

-Are TH locally regulated in response to acute pancreatitis?

To investigate whether TH are locally regulated following pancreatic damage we used the well established model of serial cerulein injections to induce pancreatitis and characterized levels of TH within the pancreas as well as in the blood during the progression of the disease. Furthermore, to investigate intracellular regulation and activity of TH we quantified expression levels of deiodinases and TH receptors genes in the pancreas and isolated acinar cells.

-What is the effect of acinar specific conditional KO of DIO3?

Mice with an acinar cell specific conditional KO for DIO3 were generated by crossing $DIO3^{fl/fl}$ and ELA-$CreERT2^{Tg/+}$ strains. To investigate the putative effect of DIO3 deletion on pancreatic homeostasis we characterize pancreatic specimens harvested from healthy mice immediately after induction of recombination via tamoxifen treatment as well as one month post-tamoxifen. Next, we compared the response to cerulein induced acute pancreatitis

between transgenic and control mice. Based on the reported observations that TH promote acinar cell proliferation, we focused on the characterization of acinar cell regeneration; for this reason, level of acinar cell proliferation was determined by immunohistochemistry (IHC) as well as gene expression of cell cycle regulators (i.e. cyclins). Furthermore, we investigated if acinar cell deletion of DIO3 alters the degree of damage and inflammation; to this aim, we measured Amylase and Lipase levels in the serum, two common markers of acinar damage, and quantified infiltrating cells within the pancreas.

-Is pancreatic regeneration altered during thyrotoxicosis or hypothyroidism?

We questioned the effect of systemic alterations of TH during pancreatic regeneration: thyrotoxicosis was induced by T3 injections whereas hypothyroidism by supplementation of sodium perchlorate and methimazole by drinking water. As mentioned above, we focused our analysis on the regenerative potential of acinar cells and characterized levels of damage and inflammation at the onset and during the progression of the disease.

-What are the molecular mechanisms of T3 driven acinar cell proliferation?

Analyses of the questions reported above brought us important results on the function of TH in pancreatic regeneration; for these reasons, we decided to extend our study and investigate the molecular mechanisms of T3-driven acinar cell proliferation. To this aim, we injected T3 to healthy mice in order to avoid the presence of damage and inflammation. We tested dose and time dependence of T3 induction of proliferation; furthermore, we determined the activation of selected signaling candidates, described in the literature to modulate acinar cell proliferation *in vivo*. Finally, we tested the putative involvement of HDAC, Akt, and TGF-β pathways; to this aim, we blocked each of these targets by either pharmacological inhibition or genetically modified mouse models.

4. Manuscript A

Local hyperthyroidism promotes acinar cell proliferation during acute pancreatitis

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Contribution: This paper represents a major part of my PhD work; I performed most of the experiments and the data analysis. Moreover, I drafted and contributed to revising the paper.

Abstract

Regeneration of the exocrine pancreas is a critical process in the pathophysiology of pancreatic diseases, as limited or defective regeneration is associated with organ dysfunction and patient morbidity. In this context, elucidating the signalling pathways that trigger and sustain pancreatic regeneration is pivotal to develop therapeutic interventions promoting the regenerative process.

In this study, we discovered that levels of thyroid hormones and deiodinases enzymes, which modify the biological activity of thyroid hormones, increased in the pancreas upon induction of acute pancreatitis. This transient increase of regional thyroid hormone availability was required to promote proliferation of pancreatic acinar cells, and consequent pancreatic regeneration. By using genetic approaches to locally increase the levels of the biologically active thyroid hormone 3,3',5-triodo-L-thyronine (T3) in the pancreas, we could show that endogenous T3 acts as mitogens for acinar cells without affecting the extent of tissue damage or the development of inflammatory infiltration. Moreover, by manipulating pharmacologically the systemic levels of thyroid hormones, we demonstrated not only that decreased levels of circulating thyroid hormones impair acinar proliferation but also that exogenous administration of T3 is effective in boosting acinar cell proliferation upon induction of pancreatitis.

In search of the molecular mechanisms underlying the observed phenotype, we showed that the mitogenic effect of T3 on acinar cells is the result of a tightly controlled and concerted action of different signaling pathways, including histone deacetylase, Akt, and TGFβ signaling, which regulated acinar proliferation in a positive and negative manner.

In conclusion, our data suggest that local availability of thyroid hormones in the pancreas is required to promote acinar cell proliferation. In addition, we propose that thyroid hormone signaling could be exploited to develop therapeutic regimens aiming at enhancing pancreatic regeneration.

Introduction

Acute pancreatitis, a debilitating inflammatory disease of the pancreas, is a major cause of gastrointestinal hospital admission [1]. The injury of the organ consequent the development of the disease leads to dysfunction and, in its severe forms, necrosis of exocrine pancreatic tissue, resulting in high morbidity and mortality. In this context, boosting regeneration of the injured pancreas is a key therapeutic target to restore the activity of the organ and limit the pathological implications associated with impaired pancreatic function.

In this work, we aimed to identify molecular factors that act as mitogens for pancreatic acinar cells and thus have the potential to be exploited therapeutically to promote pancreatic regeneration. Pancreatic acinar cells are ideal candidates to sustain the recovery of the organ following inflammatory insult, as not only constitute the vast majority of the exocrine pancreatic tissue, but also harbor the potential to proliferate in their adult state. Indeed, fully differentiated acinar cells, committed to the synthesis of digestive enzymes, are able to transiently de-differentiate to a progenitor-like state and re-enter a cell cycle program in response to pancreatitis [2]. However, this proliferative ability, reported in humans and animal models, is often limited, thus hampering the regeneration capacity of the pancreas. In recent years multiple signaling pathways and transcription factors have been described to regulate acinar cell proliferation following pancreatitis (reviewed in [3]). These studies revealed a complex network of signals that sustain cell de-differentiation or promote re-differentiation of acinar cells after injury. Importantly, these works highlight the concept that the molecular factors that drive the process of regeneration in the adult pancreas are also implicated in the developmental program of the organ. However, our knowledge regarding molecules that trigger or sustain proliferation of acinar cells is still limited. In this study, we investigated whether a proliferative stimulus to promote acinar cell proliferation is provided by a local increase of thyroid hormone levels in the pancreas upon induction of pancreatitis. The hypothesis of local hyperthyroidism to support pancreatic regeneration was built on independent lines of evidences. Firstly, consistent with the fact that a developmental program is re-activated during pancreatic regeneration, thyroid hormones play a functional role in the development of pancreatic cells during organogenesis, demonstrated in both amphibian and mammalian pancreas (reviewed in [4]). Secondly, adult acinar cells harbor the molecular machinery to respond to thyroid hormones and start proliferating when triiodothyronine (T3), the biologically active thyroid hormone that binds to thyroid hormone receptors [5], is administered exogenously [6, 7]. In this context, previous studies using a selective receptor agonist identified TRβ as the critical isoform of thyroid hormone receptor that promotes proliferation of pancreatic acinar cells upon T3 administration [8]. Finally, thyroid hormones act as mitogens during regeneration of liver [9], an organ that shares the same embryological lineage with the pancreas.

To test our hypothesis that thyroid hormones are endogenous mitogens for pancreatic acinar cells during pancreatitis, we utilized genetic and pharmacological approaches to alter local and systemic levels of thyroid hormones, and assessed acinar proliferation following cerulein-induced pancreatitis, the most widespread experimental method to generate the disease in rodents [3].

Materials and methods

Animal experiments

All animal experiments were conducted in accordance with Swiss federal animal regulations and approved by the cantonal veterinary office of Zurich. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. Mice used in this study were adult 8–14 week old male C57BL/6 mice (Envigo, Itingen, Switzerland), conditional TGFβ receptor II deficient mice (TGF-RII KO) [10] bred in our facility, and mice with inducible ablation of Deiodinase 3 in acinar cells ($ELA^{CreERT2}/D3^{flox/flox}$, D3KO) bred in our facility. D3KO animals were generated by crossing mice harboring elastase (ELA) promoter driven Cre transgene $ELA^{CreERT2Tg/+}$ [11] with transgenic mice expressing Deiodinase 3 (D3) $^{flox/flox}$ [12]. Tamoxifen (TAM -SPEC)-driven recombination was induced with 100 µL injection of 20 mg/mL tamoxifen (Sigma-Aldrich) daily for 5 consecutive days as described [13]. The CRE-negative littermates were used as controls.

Acute pancreatitis was induced via six intraperitoneal (i.p.) injections of 50 µg/kg cerulein, administered hourly on two consecutive days. Control animals received 0.9% NaCl injections. Hyperthyroidism was induced with daily injections of 400µg/Kg T3. Control animals received vehicle 0.1M NaOH, pH 7.4 with 0.5%BSA injections.

Hypothyroidism was induced by supplementation of 1% $NaClO_4$ and 0.1% methimazole (MMI) by drinking water for 28 days [14].

Class I HDAC inhibitor MS-275 (Selleckchem, Houston, USA) was injected daily i.p. at 20 mg/kg, starting one day before the first T3 injection (See scheme Fig.S4).

Akt inhibitor, MK-2206 (A3010, APExBIO) was injected four times daily i.p. at 8 mg/kg, starting before the first T3 injection (see scheme Fig.S5).

Mice were anesthetized by isoflurane inhalation. The harvest times for the different experiments are expressed as hours after the first cerulein or T3 injection and are specified in the individual figure panels.

Groups of 5 animals were tested for each experimental condition. Animals were assigned randomly to different experimental groups for all in vivo studies. Data collection and evaluation of all experiments were performed blinded to the group identity.

Primary acini were isolated from C57BL/6 mice 24h after induction of pancreatitis via collagenase digestion according to [15].

Immunohistochemistry

Pancreas specimens were embedded in paraffin for histological analyses as described [16]. Primary antibodies used in this study were: rabbit anti-Ki67 (#ab16667, Abcam, Cambridge, UK, 1:200); rabbit anti-phospho-H3(06-570, Millipore) rabbit anti-amylase (#A8273-1VL, Sigma-Aldrich, Buchs, Switzerland, 1:1000); rabbit anti-PU.1 (#2266, Cell Signaling Technologies, Danvers, MA, 1:200); rabbit anti-phospho-histone H2A.X (Ser139) (#9718, Cell Signaling Technologies, Danvers, MA, 1:500); rabbit anti-cleaved Caspase-3 (Asp175) (#9661, Cell Signaling Technologies, Danvers, MA, 1:1500). Secondary antibodies used in this study were biotinylated goat anti-rabbit IgG (H + L), included in the Vectastain® ABC Kit (PK-4001, Vector Laboratories, Peterborough, UK). All staining, with the exception of Amylase, were performed with a DAKO autostainer Link 48.

Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×200) per slide using the NIS Elements BR Analysis and Cell^P analysis software. Number of positive cells was normalized on the area occupied by pancreatic acinar cells present in each power field. Pancreatic ducts, islets and vessels were excluded from the analysis.

Quantitative analysis of ADM was performed as described in [17]. Briefly, paraffin-embedded pancreas specimens were immunostained for amylase, slides were scanned with a NDP NanoZoomer Digital Pathology Slide Scanner (Hamamatsu) and analyzed for ADM lesions in a blinded fashion. ADM present in the entire pancreas slide of 5 mice from each treatment condition were quantified by manual counting. ADM were identified according to: i) loss of amylase content, ii) structural re-organization into tubular complexes, iii) stromal reaction characterized by presence of cell infiltrates. The area occupied by ADM was expressed as percentage of total pancreatic area present in each slide.

Biochemical analyses

Levels of enzymatic activity of amylase and lipase were measured in blood serum collected via heart puncture. Enzyme activities were measured using the Fuji Dri-Chem 4000i analyzer (FUJIFILM Corporation, Tokyo, Japan).

Blood levels of F-T4 and F-T3 were quantified with analyzer Cobas 8000 Modul-System e 602 (Roche), according to the manufacturer instruction.

Tissue levels of F-T3 were measured using an Elisa Kit (CUSABIO Technology LLC, Houston, TX, USA) according to the manufacturer instruction.

Nuclear protein extraction and HDAC activity

Nuclear proteins were extracted from 20 mg of pancreatic tissue with the EpiQuik™ Nuclear Extraction Kit (Epigentek Group Inc, Mountain View, CA) and HDAC activity was measured in the nuclear extracts with the fluorimetric EpiQuik HDAC Activity/Inhibition Assay Kit (Epigentek Group Inc. Mountain View, CA), following the manufacturer's instructions.

Western blotting

20 mg of pancreatic tissue was homogenized in RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by a Bradford protein assay (BioRad, Hercules, CA, USA). 20 μg of proteins were resolved by SDS-PAGE electrophoresis and blotted onto nitrocellulose membranes using a V3 Western Workflow system (BioRAd, Hercules, CA, USA) according to the manufacturer's protocols.

Membranes were incubated with primary antibodies overnight at 4°C. Primary antibodies used in this study were: p-AKT (4051, Cell Signaling), AKT (9272, Cell Signaling); rabbit anti α-tubulin (2125, Cell Signaling); p-Smad2/3 (Ser423/425) (8828, Cell Signaling); SMAD2/3 (8685, Cell Signaling).

Transcript analysis

Total RNA was extracted from pancreatic tissue, as described previously [18], and RNA quality control was performed by RIN (RNA Integrity Number) measurement using a 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). RNA was reverse-transcribed with qScript™ cDNA SuperMix (Quanta Biosciences, Beverly, CA, USA). Gene expression was measured by real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City,

USA) using Taqman probes (Applied Biosystems, Foster City, CA, USA). Transcript levels were normalized using 18S RNA as a reference and expressed as $2^{-\Delta\Delta Ct}$ relative to the value of control animals.

Statistics

Groups of 5 animals were tested for each experimental group. The data are expressed as means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test or one-way analysis of variance followed by Dunnett post test (GraphPad Prism 7; GraphPad Software, Inc.) and a probability value <0.05 was considered statistically significant.

Results

Local hyperthyroidism is induced in the pancreas during acute pancreatitis

To test whether thyroid hormones act as mitogens during pancreatic regeneration upon inflammatory injury, we first investigated whether T3 levels increase in the pancreas following induction of acute pancreatitis with serial injections of cerulein. Cerulein administration (scheme depicted in **Fig.1A**) results in a time-dependent increase in acinar cell proliferation, as quantified by the levels of acinar cells positive for the general proliferation marker Ki67 (**Fig.1B**). Interestingly, we observed a transient surge of free T3 (F-T3) levels, the biologically active form of the hormone (reviewed in [5]), in the pancreas (**Fig.1C**), the kinetic of which preceded the peak of acinar cell proliferation. Conversely, levels of thyroid hormones F-T4 and F-T3 transiently decreased in blood (**Fig.1D**). Increased F-T3 levels in pancreatic tissue may derive from increased uptake from the circulation of either T3 and/or of T4, with subsequent conversion into T3 by the action of deiodinases (DIO) enzymes, which regulate the intracellular hormone concentration in a time- and tissue-specific fashion, independently on the levels present in blood [5]. We detected an early up-regulation of DIO2, the enzyme responsible for local T4 to T3 conversion in target cells [19], in the pancreas in response to induction of pancreatitis (**Fig.1E**). Importantly, DIO3, responsible for inactivating both T4 and T3 hormones, was also up-regulated in the pancreas, while DIO1 expression was transiently down-regulated. DIO2 and DIO3 up-regulation was also detected in pancreatic acinar cells isolated after 24h of cerulein treatment (**Fig.1F**), suggesting that acinar cells are able to regulate the intracellular concentration of thyroid hormones. Analyses of DIO expression in multiple organs revealed that DIO regulation was either detected exclusively (DIO1, 2) or showing the highest regulation (DIO3) in the injured pancreas (**Fig.1G**). Collectively, these data support the hypothesis that increased levels of F-T3 and consequent local hyperthyroidism are specifically and transiently induced in the organ in response to acute pancreatitis.

Genetic ablation of DIO3 in acinar cells increases acinar proliferation during acute pancreatitis

To test whether the transient hyperthyroidism observed upon induction of pancreatitis promotes acinar proliferation, we increased the levels of thyroid hormones in acinar cells by knocking out DIO3 in these cells using a tamoxifen-inducible approach (scheme depicted in **Fig.2A**). In the absence of pancreatitis, DIO3 conditional KO (D3KO) mice presented a normal phenotype indistinguishable from control mice. This was evident by normal pancreatic histology (**Fig.2B**), lack of acinar damage (**Fig.S1A**), or development of inflammation (**Fig.2C**). Similarly,

no differences in basal levels of acinar cell proliferation were observed between transgenic and control mice (**Fig.2D**), even one month after tamoxifen treatment (Fig.S1B), suggesting that ablation of DIO3 does not compromise pancreatic homeostasis in untreated mice. Similarly, levels of pancreatic and circulating thyroid hormones were not altered in D3KO mice in untreated conditions (**Fig.2E**). However, upon induction of acute pancreatitis (treatment scheme depicted in **Fig.2F**), proliferation of acinar cells was higher in D3KO animals, as determined by number of Ki67 positive cells (**Fig. 2G**) and cyclin expression in the pancreas (**Fig.2H**). Increased acinar proliferation in D3KO animals was preceded by increased levels of free-T3 in the pancreas (**Fig. 2I**). Moreover, increased acinar proliferation was not a consequence of increased pancreatic injury upon induction of pancreatitis, as blood levels of amylase and lipase, the most reliable indicators of acinar cell damage, were comparable in control and D3KO mice (**Fig.2J**). Similarly, it was not driven by increased recruitment of PU.1-positive inflammatory cells, as their number was comparable in the two strains (Fig.2K). Finally, increased acinar proliferation was not accompanied by changes in the levels of acinar-to-ductal metaplasia (**Fig.2L**), a transient de-differentiation of acinar cells observed in the tissue during pancreatic regeneration. Collectively, these data indicate that ablation of DIO3 in acinar cells is sufficient to increase pancreatic levels of F-T3 and enhance acinar proliferation upon induction of acute pancreatitis.

Systemic alterations of thyroid hormone levels modulate acinar cell proliferation during acute pancreatitis

After showing that local hyperthyroidism induced in the pancreas following acute pancreatitis supports acinar proliferation, we then investigated whether systemic alteration of thyroid hormone levels affect the extent of acinar proliferation during the disease. Systemic hyperthyroidism was induced by daily administration of T3 starting after the first set of cerulein injections, while systemic hypothyroidism was induced by administration of $NaClO_4$ and methimazole (MMI) for 28 days (regimen scheme depicted in **Fig.3A**). Analysis of thyroid hormone levels in serum confirmed the efficacy of the treatments as T3 administration increased F-T3 levels and decreased F-T4, as a consequence of negative feedback loop, while MMI administration decreased the levels of both hormones (**Fig.3B**).

T3 treatment significantly increased proliferation of acinar cells (**Fig.3C**), and cyclin expression in the pancreas (**Fig.3D**). Conversely, MMI administration decreased both acinar proliferation and cyclin expression (**Fig.3C, D**). Administration of T3 to hypothyroid mice rescued the defective acinar proliferation (**Fig.3E**), further demonstrating that increased levels of this hormone are sufficient to boost acinar cell proliferation.

Changes in acinar proliferation observed during systemic hyper- and hypothyroidism were not a consequence of changes in initial damage of acinar cells or infiltration of inflammatory cells. Indeed, manipulation of thyroid hormone levels did not modify serum levels of amylase and lipase, early indicators of acinar cell damage (**Fig.3F**). Similarly, levels of inflammatory cell infiltration, quantified by the number of PU.1-positive cells, were unchanged by induction of hyper- or hypothyroidism (**Fig.3G**). Furthermore, differences in acinar replication were not correlated to differences in acinar apoptosis or DNA damage, as shown by quantification of cleaved-caspase3 (**Fig.S2A**) and p-H2A.X (**Fig.S2B**). Collectively, these data suggest that altering the systemic levels of thyroid hormones does not modify

pancreatic sensitivity to cerulein administration and recruitment of inflammatory cells. In addition, they indicate that changes in acinar proliferation induced by systemic variation of thyroid hormone levels are independent from the levels of acinar cell injury and inflammatory cell infiltration.

Finally, to demonstrate that T3 sustains acinar cell proliferation independently from T4, we administered Iopanoic acid (IOP), a specific deiodinase inhibitor that blocks T4 to T3 conversion [20], in combination with cerulein (**Fig.3H**). Analysis of thyroid hormone levels confirmed that IOP administration decreased blood levels of F-T3, whereas F-T4 amount was unchanged (**Fig.3I**). Similar to what we observed in hypothyroid mice, IOP-treated animals showed a significant decrease in the number of proliferating acinar cells (**Fig.3J**). Overall, these data demonstrate the existence of a positive correlation between thyroid hormone levels, in particular T3 availability, and proliferation of acinar cells in response to acute pancreatitis.

Acinar proliferation induced by T3 administration is promoted by HDAC activity and Akt signaling

Next, we investigated the molecular mechanisms underlying the mitogenic action of T3 in pancreatic acinar cells. To be able to focus exclusively on T3 action without the confounding aspect of pancreatic inflammation, we supplemented T3 *in vivo* in the absence of cerulein-induced pancreatitis (scheme depicted in **Fig.4A**). Analysis of acinar cells positive for proliferation markers Ki67 and pH3 (**Fig.4B**) and pancreatic expression of early and late cyclins (**Fig.S3A**) showed that T3 exerted a profound mitogenic effect on acinar cells 96 hours after the beginning of the treatment, as previously described [6]. Moreover, T3-mediated induction of acinar proliferation was dose-dependent (**Fig.4C**) and it did not elicit inflammatory cell infiltration (**Fig.4D**) or morphological alteration of pancreatic tissue (**Fig.S3B**), which further support our observation that T3 does not influence pancreatic inflammation during the development of pancreatitis.

T3 is known to regulate multiple pathways within the cells by genomic and non-genomic actions [21]. Here we tested whether signaling pathways that are known to support acinar proliferation during pancreatitis are activated in the pancreas upon T3 administration in the absence of inflammation. We first assessed whether T3 administration triggered the upregulation of histone deacetylases (HDAC), a class of epigenetic modifiers that was recently shown to be activated during pancreatitis and promotes proliferation of acinar cells [22, 23]. T3 treatment was sufficient to increase both the expression of different HDAC isoforms (**Fig.4E**) and total HDAC activity in the pancreas (**Fig.4F**). *In vivo* treatment with the specific class I HDAC inhibitor MS-275 (treatment scheme depicted in **Fig.S4A**) effectively reduced HDAC activation (**Fig.S4B**) and reduced acinar cell proliferation (**Fig.4G**) and cyclin B expression (**Fig.S4C**) induced by T3. Inhibition of acinar cell proliferation was accompanied by increased expression of cell cycle inhibitors p15/INK4B, p16/INK4A, and p18/INK4C (**Fig.S4D**).

We then investigated whether T3 administration promoted the activation of phosphatidylinositol 3-kinase (PI3K)/Akt. The Akt pathway is important for pancreatic proliferation, as reduction of this signaling suppresses acinar cell division [24] and its constitutive activation in adult acinar cells leads to excessive proliferation and malignant transformation [25]. T3 supplementation significantly increased pancreatic gene expression of Akt 1 and 2 isoforms (**Fig.4H**), Akt protein levels and AKT activation via

phosphorylation (**Fig.4I**). To test the functional relevance of Akt signaling, we administered the potent pan-Akt inhibitor MK-2206, which efficiently reduces Akt activation *in vivo* [26]. MK-2206-treated mice (treatment scheme depicted in **Fig.S5A**) showed reduced Akt activation (**Fig.4J**), confirming the efficacy of the compound. Importantly, MK-2206 treatment strongly reduced acinar cell proliferation (**Fig.4K**) and cyclin expression (**Fig.S5B**) induced by T3. Reduced acinar cell proliferation correlated with a significant increase in the cell cycle inhibitor p15/INK4B and p16/INK4A (**Fig.S5C**), which could likely contribute to the observed inhibition of proliferation.

Collectively, these data demonstrate that T3 promotes the proliferation of healthy acinar cells by activating signaling pathways known to support acinar proliferation in the course of pancreatitis.

Acinar proliferation induced by T3 administration is restrained by TGFβ signaling.

We recently showed that increased levels of Transforming Growth Factor beta (TGFβ) and activation of TGFβ signaling in pancreatic cells by TGFβ receptor II tightly reduces acinar cell proliferation during cerulein-induced pancreatitis [10], suggesting that TGFβ signaling belongs to the arsenal of negative regulators that control the level of pancreatic regeneration. Here we tested whether TGFβ levels increased upon T3 administration (scheme depicted in **Fig.4A**) and whether TGFβ signaling limited the extent of T3-induced acinar proliferation also in the absence of an inflammatory reaction.

Expression levels of TGFβ 1–3 isoforms increased in the pancreas at 96h following T3 supplementation (**Fig.5A**). However, increased expression of TGFβ ligands was not accompanied by a robust increase in phosphorylation of SMAD effector proteins at this time point (**Fig.5B**), suggesting that activation of TGFβ signaling is blunted by the presence of T3.

To investigate whether the observed upregulation of TGFβ exerts an anti-mitogenic action, we assessed the levels of T3-induced acinar cell proliferation in mice lacking TGFβ signaling following ablation of TGFβ receptor II in the pancreas [10] (scheme depicted in **Fig.5C**). Similar to what we observed upon cerulein administration and inflammatory injury, acinar proliferation induced by T3 treatment increased in the absence of TGFβ receptor II, as quantified by the levels of proliferation markers (**Fig.5D**) and cyclin expression (**Fig.S6A**). T3 treatment did not change robustly the expression of cell cycle inhibitors (**Fig.S6B**). Overall, our data suggest that the extent of acinar cell proliferation induced upon T3 administration is restrained by TGFβ signaling.

Discussion

Thyroid hormones play essential roles in the embryonic and adult homeostasis across species, by regulating the development of several organs, growth and metabolic processes [19].

Here we showed that thyroid hormone signaling is activated during the pathological setting of pancreatitis where it promotes pancreatic regeneration.

These findings have important implication for translational application in a therapeutic setting, as defective or limited pancreatic regeneration is at the core of morbidity associated not only with acute pancreatitis but also with acinar atrophy and exocrine pancreatic insufficiency found in a broader range of pancreatic diseases, including chronic pancreatitis, diabetes and cystic fibrosis.

Our results highlight three major concepts that demonstrate the profound effect exerted by thyroid hormones in the pathophysiology of pancreatitis. Firstly, we showed that levels of F-T3, the biologically active form of thyroid hormones, transiently increased in the pancreas following inflammatory injury. This local hyperthyroidism was determined by direct quantification of the hormone in the tissue, by organ-specific up-regulation of enzymes responsible for thyroid hormone synthesis (reported also in a recently transcriptome-based study [27]) and by up-regulation of thyroid hormone inactivating enzyme deiodinase 3, an accurate functional marker of thyroid hormone status [28, 29]. The increase of T3 levels in the injured pancreas was independent from circulating levels of thyroid hormones, which decreased in the initial stages of the disease. This suggests that serum hormone levels are not a direct measure of the intracellular T3 availability in the organ. In addition, the transient nature of T3 increase in the organ implies that the concentration and consequent signaling of the hormone is controlled by the activity of both activating and inactivating deiodinases engaged in a time-dependent manner in the tissue, as further supported by our deiodinases expression data.

Secondly, we revealed that selectively increasing T3 levels in the pancreas, by reducing deiodinase 3-dependent T3 inactivation, boosted acinar proliferation in the context of pancreatic injury. This mitogenic effect was exquisitely acinar-specific, as it did not affect replication of non-acinar cells and was not accompanied by changes in tissue damage, infiltration of inflammatory cells, or trans-differentiation of acinar cells into ADM. In addition, we showed that deiodinase 3 ablation in untreated conditions did not increase T3 levels in the pancreas nor induced proliferation of acinar cells, suggesting that an initial pancreatic damage is necessary to trigger local hyperthyroidism and its consequent mitogenic effects.

Thirdly, we demonstrated that manipulating the circulating levels of thyroid hormones had a direct impact on acinar cell proliferation. This raises the important clinical implication that hypothyroid patients may suffer from impaired pancreatic regeneration in the context of pancreatitis. This potential concern complement previous reports showing that low thyroid hormone levels in acute pancreatitis patients are a marker for increased severity of the disease [30, 31].

Collectively, our data showing enhanced acinar proliferation induced by both tissue specific and systemic elevation of thyroid hormones provide a proof of concept for a potential therapeutic target to improve organ regeneration. While systemic T3 administration did not provoke adverse outcomes of tissue damage or inflammation (our study and [6, 7]), T3 exerts mitogenic effects in different organs. Thus, future research should focus on the development of therapeutic interventions promoting proliferation of acinar cells in a selective manner during the regenerative phase of the organ. In this context, our data suggest that inhibition of deiodinase 3 activity is an appealing target to increase T3 levels locally in the pancreas, as its expression was greatly up-regulated in the injured organ and its ablation was sufficient to increase acinar proliferation.

An additional focus of our study was to identify molecular pathways activated during T3-induced acinar proliferation. By using the controlled setting of T3 administration in the absence of pancreatitis, we demonstrated that T3 triggers the production of several signaling molecules, the activity of which either promotes or reduces acinar proliferation. This array of regulatory mechanisms provides the underlining explanation for the fact that T3-induced

acinar proliferation is a temporally controlled process. Importantly, they revealed that the signaling pathways activated by T3 are also crucial in regulating acinar proliferation in the context of pancreatitis [10, 23, 24], further supporting the concept that T3 is a major factor in driving acinar cell division upon inflammatory insult.

Amongst the pathways identified, we discovered that T3-induced acinar proliferation is promoted by the activity of the epigenetic modifier class I HDACs and by Akt signaling. HDACs are known members of the thyroid receptor co-repressor complex regulating the genomic effects of T3-induced transcription [32], while Akt signaling is considered an example of non-genomic actions of thyroid hormones, which are rapid in their onset and independent from nuclear uptake of T3 (reviewed in [21, 33]). Thus, the dual activation of genomic and non-genomic pathways suggests that both modality of T3 signaling are engaged in the pancreas to drive acinar proliferation. This is consistent with Akt contributing to mediate the effects of T3 administration reported in other tissues and cells, including skeletal muscle [34], heart [35] and fibroblasts [36], suggesting that activation of non-genomic actions is a common pathway in T3-mediated signaling.

The third signaling pathways we identified in the pancreas in response to T3 administration is TGFβ. Interestingly, while TGFβ isoforms were upregulated during T3 treatment, activation of TGFβ signaling was blunted. This in agreement with a previous report showing that T3 antagonizes TGFβ signaling by reducing Smad phosphorylation [37]. The functional relevance of this signaling inhibition is likely to promote acinar proliferation, as defective TGFβ receptor potentiated the mitogenic effect of T3 in acinar cells. In this context, it is tempting to speculate the existence of a negative feedback loop where TGFβ is upregulated as a consequence of T3-driven proliferation and it acts as a repressor of cell division. Interestingly, the cross-talk between T3 and TGFβ is not always of antagonistic nature. As an example, T3 administration activates TGFβ signaling in hepatocellular carcinoma and results in reduced cell proliferation [38].

Conclusions

Based on our collective results, we propose that establishment of a time-dependent and tightly controlled local hyperthyroidism in the pancreas supports acinar proliferation following injury. This discovery harbors therapeutic implications and highlights the potential of interventional strategies based on the modulation of thyroid hormonal signal to promote pancreatic regeneration.

The mitogenic effect exerted by T3 in adult pancreatic acinar cells is shared by several tissues and cell types, including renal proximal tubular epithelial cells [7], heart cells [39], liver cells [40-42].

However, a different situation is observed during muscle regeneration, where T3 is required to differentiate the local population of satellite stem cells into mature muscle cells [12]. Similarly, low levels of thyroid hormones maintain the renewal capacity of oligodendrocyte progenitor cells, while their elevation promotes the switch from stem cell proliferation to cell differentiation [43]. Intriguingly, but not entirely surprisingly given the pivotal role of cancer stem cells to self-renew and drive tumorigenesis [44], low levels of thyroid hormones are required to support the development of different malignancies (reviewed in [45]).

Thus, our results contribute to expand the concept that thyroid hormones induce different outcomes in

different replicative contexts where they control the balance between proliferation and differentiation.

While this study provides the first insight into endocrine regulation of acinar proliferation, further studies are required to elucidate fully the details of thyroid hormone actions in the pancreas. An open question not addressed in our study is the identification of the thyroid hormone receptors involved in the observed phenotype and the characterization of their cistromes *in vivo*. This is of special interest as the receptor isoforms have different repertoires of target genes [46], the expression of which can be affected by thyroid hormone binding in either a positive or negative manner [47].

Another open question concerns the role of transmembrane transporters for thyroid hormones [48, 49] in regulating hormone availability in the pancreas. While a thorough characterization of transporter families has not been performed in acinar cells, it is worth mentioning that amongst the L-type amino acid transporters involved in thyroid hormone uptake, Lat1 and Lat2 are expressed on the basolateral membrane of adult acinar cells [50].

Finally, the investigation of the upstream signals that regulate expression of deiodinases in the injured pancreas is of major interest. Their identification is crucial not only to understand how the onset of local hyperthyroidism takes place, but also to identify possible targets to exploit as therapeutic intervention.

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Figure legends

Figure 1. Pancreatic levels of F-T3 increase following cerulein-induced pancreatitis. (A) Schematic representation of cerulein (Cer) administration. Mice received six injections daily over two consecutive days. Harvest time is indicated as hours after the first cerulein injection. **(B)** Quantification of acinar cells positive for the replication marker Ki67 at the indicated time after cerulein administration. Right panels, representative microphotographs of stained cells. **(C)** Quantification of free T3 (F-T3) in the pancreas at the indicated time after cerulein administration. **(D)** Quantification of free T4 and free T3 in the mice sera at the indicated time after cerulein administration. **(E)** qPCR of deiodinase (DIO) 1-3 in the pancreas at the indicated time after cerulein administration. **(F)** qPCR of deiodinase (DIO) 1-3 in pancreatic acini isolated 24 hours after cerulein administration. Right panel, representative microphotographs of isolated acini. **(G)** qPCR of deiodinase (DIO) 1-3 in different organs at the indicated time after cerulein administration. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

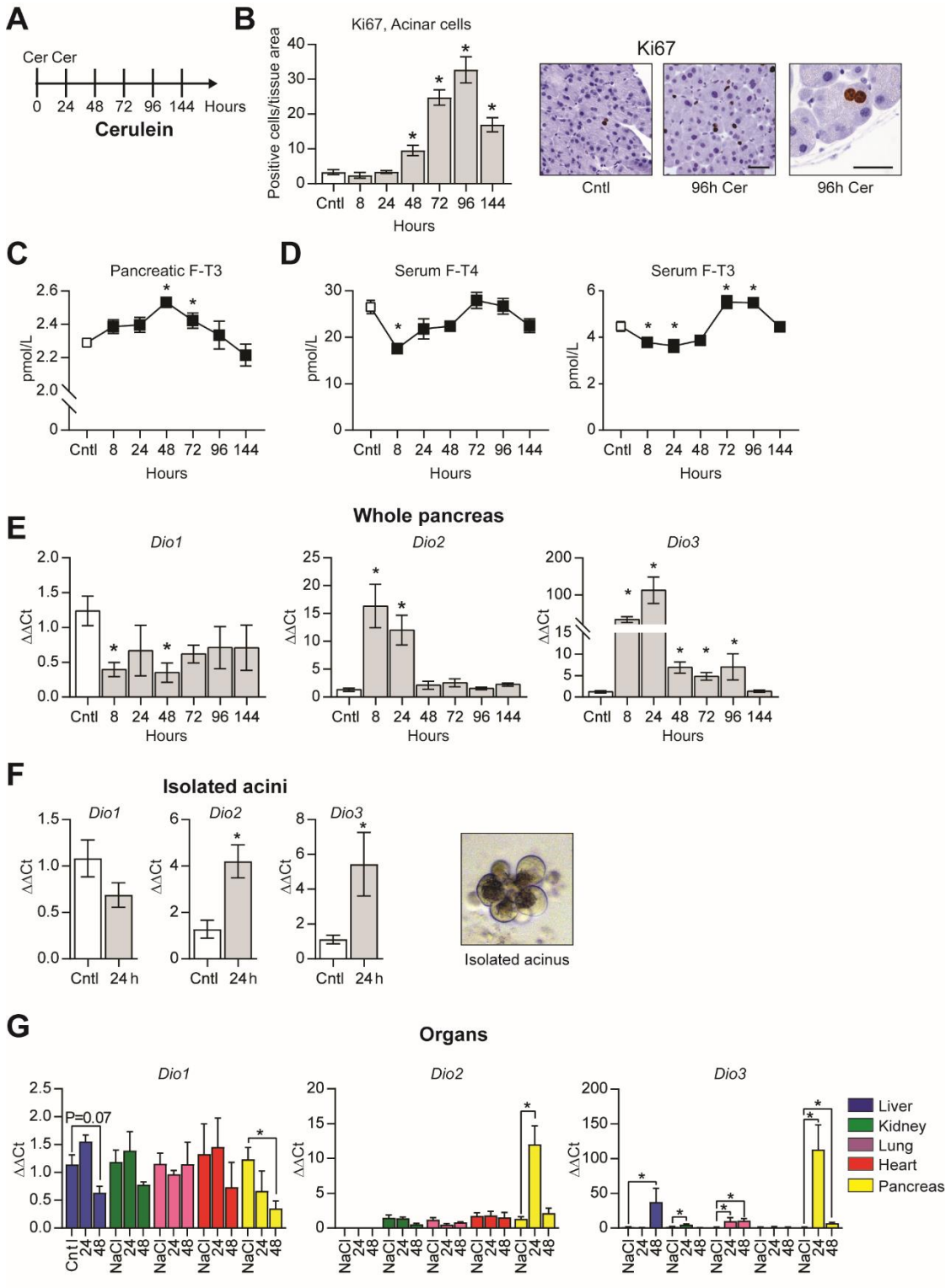
Figure 2. Ablation of deiodinase 3 in acinar cells increases acinar proliferation following induction of pancreatitis. (A) Schematic representation of conditional deiodinase 3 knocked out breeding. **(B)** Hematoxylin and Eosin (H&E) staining of pancreata in wild type (WT) and deiodinase 3 knocked out (D3KO) mice in untreated conditions. **(C)** Quantification of PU.1-positive inflammatory cells in untreated WT and D3KO mice. Right panels, representative microphotographs of stained cells (arrows). **(D)** Quantification of Ki67-positive acinar and interstitial cells in untreated mice. Right panels, representative microphotographs of stained acinar (arrows) and interstitial (arrowheads) cells). **(E)** Quantification of pancreatic and serum levels of free T3 (F-T3) and free T4 (F-T4) in untreated mice. **(F)** Schematic representation of induction of pancreatitis with cerulein injections in WT and D3KO mice. **(G)** Quantification of Ki67-positive acinar cells at the indicated time following induction of pancreatitis. Right panels, representative microphotographs of stained cells 96h after pancreatitis induction. **(H)** qPCR of cyclin expression in the pancreas at the indicated time following induction of pancreatitis. **(I)** Quantification of free T3 in the pancreas at the indicated time following induction of pancreatitis. **(J)** Quantification of amylase and lipase activity in serum at the indicated time following induction of pancreatitis. **(K)** Quantification of PU.1-positive inflammatory cells at the indicated time after induction of pancreatitis. Right panels, representative microphotographs of stained cells (arrows). **(L)** Quantification of ADM 96 hours after induction of pancreatitis. Right panel, representative microphotograph of ADM area (asterisk). Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 3. Systemic alteration of thyroid hormone levels affects acinar proliferation following induction of pancreatitis. (A) Schematic representation of pancreatitis induction upon hyperthyroidism (i) and hypothyroidism (II) with T3 and MMI administration, respectively. **(B)** Quantification of serum levels of free T4 (F-T4) and free T3 (F-T3) 96 hours after induction of pancreatitis. **(C)** Quantification of Ki67 and pH3-positive acinar cells 72 and 96 hours after induction of pancreatitis. Right panels, representative microphotographs of stained cells 96 hours after pancreatitis induction. **(D)** qPCR of cyclin expression in the pancreas at the indicated time following induction of pancreatitis. **(E)** Quantification of Ki67-positive acinar cells 96 hours after induction of pancreatitis.

(F) Quantification of amylase and lipase activity in serum at the indicated time following induction of pancreatitis. **(G)** Quantification of Pu.1-positive inflammatory cells at the indicated time after induction of pancreatitis. **(H)** Schematic representation of pancreatitis induction upon IOP treatment. **(I)** Quantification of serum levels of free T4 (F-T4) and free T3 (F-T3) at the indicated time after induction of pancreatitis. **(J)** Quantification of Ki67 and pH3-positive acinar cells at the indicated time after induction of pancreatitis. Right panels, representative microphotographs of stained cells 96 hours after induction of pancreatitis. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 4. T3-induced acinar proliferation is supported by HDAC activity and Akt signaling. (A) Schematic representation of T3 treatment. **(B)** Quantification of Ki67 and pH3-positive acinar cells 96 hours after T3 treatment. **(C)** Quantification of Ki67-positive acinar cells 96 hours after T3 treatment at the indicated concentrations. **(D)** Quantification of PU.1-positive inflammatory infiltrating the pancreas 96 hours after T3 treatment. **(E)** qPCR of class I and class II HDAC expression in the pancreas 96 hours after T3 treatment. **(F)** Quantification of HDAC activity in the pancreas 96 hours after T3 treatment. **(G)** Quantification of Ki67 and pH3-positive acinar cells 96 hours after T3 treatment in the presence of the selective class I HDAC inhibitor MS-275 (MS). Right panels, representative microphotographs of stained cells. **(H)** qPCR of Akt isoform expression in the pancreas 96 hours after T3 treatment. **(I)** Western blot analyses of Akt expression and activation in the pancreas 96 hours after T3 treatment. **(J)** Western blot analyses of Akt activation in the pancreas 96 hours after T3 treatment in the presence of the selective Akt inhibitor MK-2206 (MK). **(K)** Quantification of Ki67 and pH3-positive acinar cells 96 hours after T3 treatment in the presence of the selective Akt inhibitor MK-2206 (MK). Right panels, representative microphotographs of stained cells. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 5. T3-induced acinar proliferation is restrained by TGFβ signaling. (A) qPCR of TGFβ isoform expression in the pancreas 96 hours after T3 treatment. **(B)** Western blot analyses of SMAD expression and activation in the pancreas 96 hours after T3 treatment. **(C)** Schematic representation of T3 treatment in wild type (WT) and TGFβ receptor II knocked out mice (KO). **(D)** Quantification of Ki67 and pH3-positive acinar cells 96 hours after T3 treatment in WT and KO mice. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.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Cer Cer
0 24 48 72 96 144 Hours
Cerulein
B
Ki67, Acinar cells
Positive cells/tissue area
Cntl 8 24 48 72 96 144
Hours
Ki67
Cntl
96h Cer
96h Cer
C
Pancreatic F-T3
pmol/L
Cntl 8 24 48 72 96 144
Hours
D
Serum F-T4
pmol/L
Cntl 8 24 48 72 96 144
Hours
Serum F-T3
pmol/L
Cntl 8 24 48 72 96 144
Hours
E
Whole pancreas
Dio1
Dio2
Dio3
ΔΔCt
Cntl 8 24 48 72 96 144
Hours
F
Isolated acini
Dio1
Dio2
Dio3
ΔΔCt
Cntl 24 h
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G
Organs
Dio1
Dio2
Dio3
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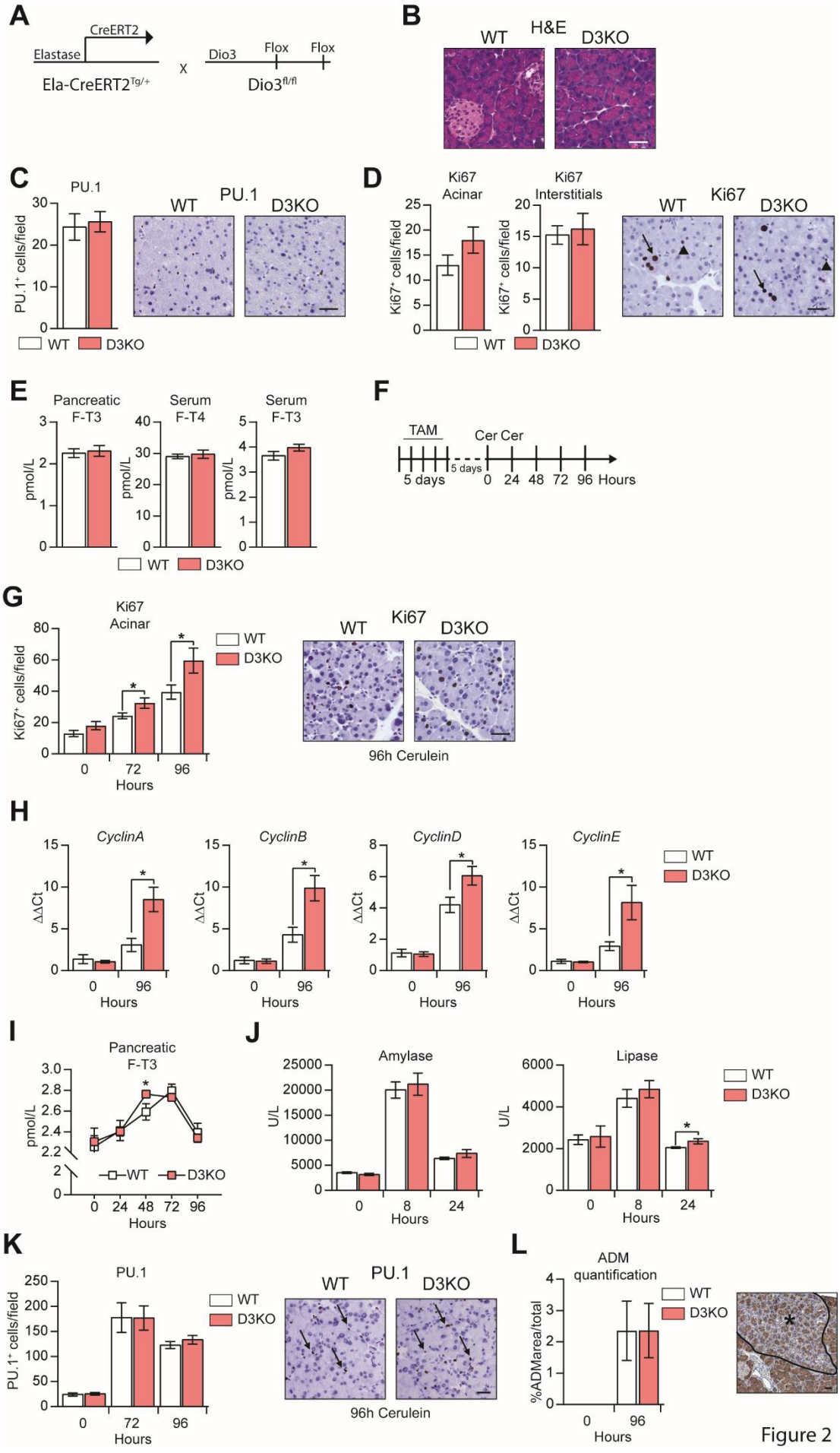
Kidney
Lung
Heart
Pancreas

Figure 1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CreERT2
Elastase
X
Dio3
Flox
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Ela-CreERT2Tg/+
Dio3fl/fl
B
H&E
WT
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C
PU.1
PU.1+ cells/field
WT
D3KO
PU.1
D
Ki67
Acinar
Interstitials
Ki67+ cells/field
WT
D3KO
Ki67
E
Pancreatic
F-T3
Serum
F-T4
Serum
F-T3
pmol/L
WT
D3KO
F
TAM
5 days
Cer Cer
5 days
0 24 48 72 96 Hours
G
Ki67
Acinar
Ki67+ cells/field
WT
D3KO
0 72 96
Hours
Ki67
96h Cerulein
H
CyclinA
CyclinB
CyclinD
CyclinE
ΔΔCt
0 96
Hours
WT
D3KO
I
Pancreatic
F-T3
pmol/L
WT
D3KO
0 24 48 72 96
Hours
J
Amylase
Lipase
U/L
0 8 24
Hours
WT
D3KO
K
PU.1
PU.1+ cells/field
WT
D3KO
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Hours
PU.1
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L
ADM
quantification
%ADMarea/total
WT
D3KO
0 96
Hours

Figure 2

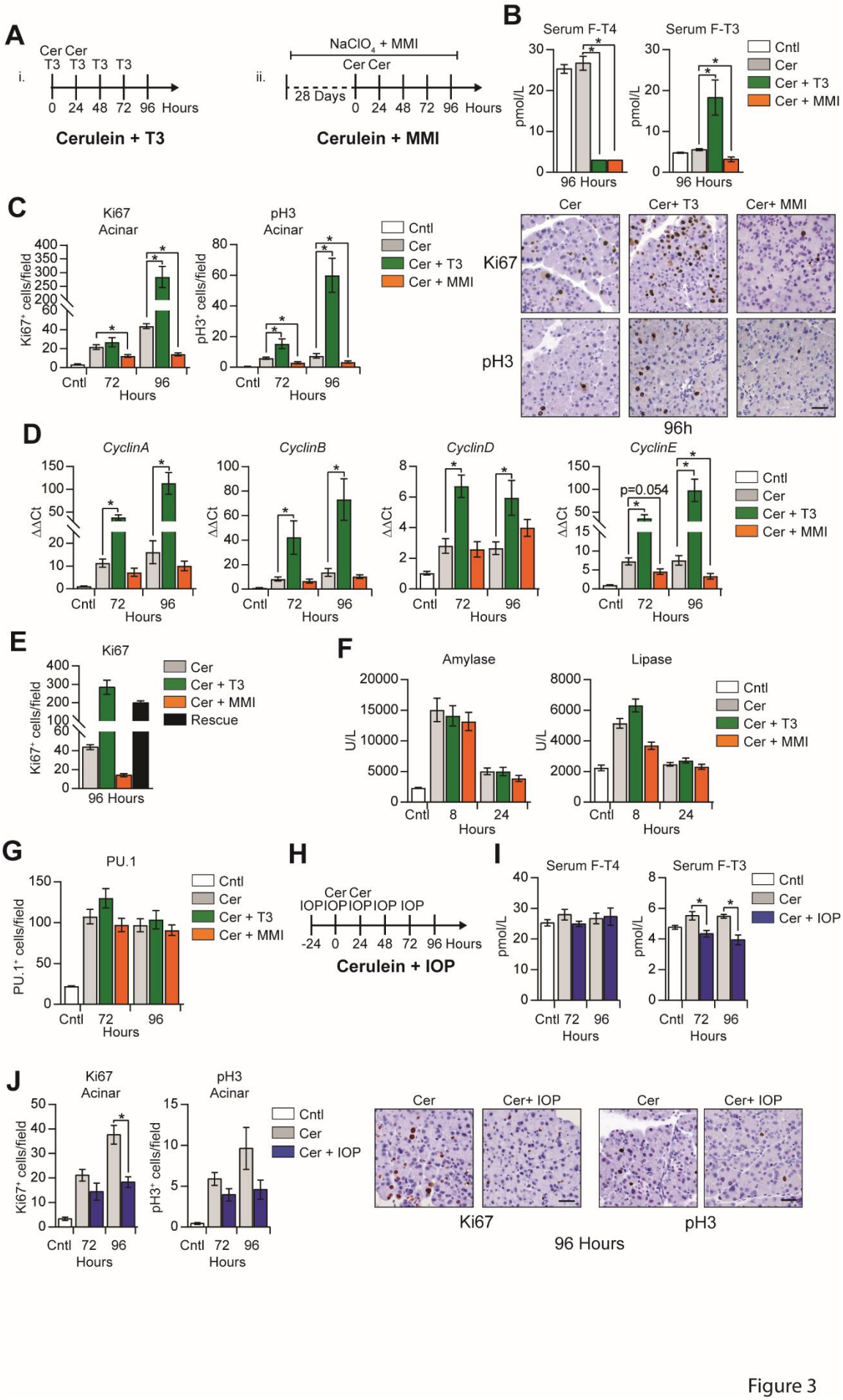
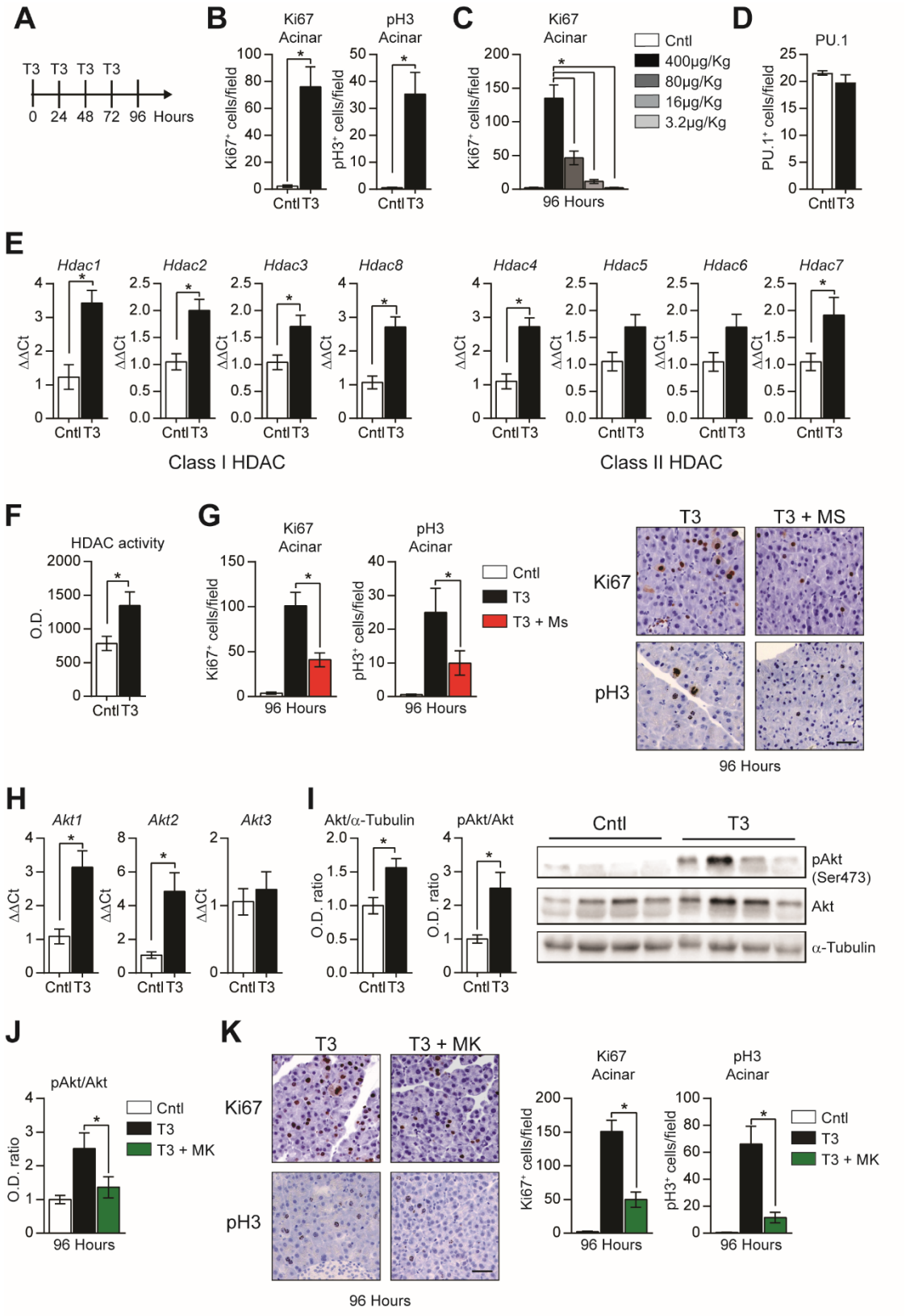

A
i.
Cer Cer
T3 T3 T3 T3
0 24 48 72 96 Hours
Cerulein + T3
ii.
NaClO4 + MMI
Cer Cer
28 Days
0 24 48 72 96 Hours
Cerulein + MMI
B
Serum F-T4
Serum F-T3
pmol/L
96 Hours
Cntl
Cer
Cer + T3
Cer + MMI
C
Ki67
Acinar
pH3
Acinar
Ki67+ cells/field
pH3+ cells/field
Cntl 72 96
Hours
Cer
Cer+ T3
Cer+ MMI
Ki67
pH3
96h
D
CyclinA
CyclinB
CyclinD
CyclinE
ΔΔCt
p=0.054
Cntl 72 96
Hours
E
Ki67
Ki67+ cells/field
96 Hours
Rescue
F
Amylase
Lipase
U/L
Cntl 8 24
Hours
G
PU.1
PU.1+ cells/field
Cntl 72 96
Hours
H
Cer Cer
IOP IOP IOP IOP IOP
-24 0 24 48 72 96 Hours
Cerulein + IOP
I
Serum F-T4
Serum F-T3
pmol/L
Cer + IOP
J
Ki67
Acinar
pH3
Acinar
Ki67+ cells/field
pH3+ cells/field
Cer
Cer+ IOP
Ki67
pH3
96 Hours

Figure 3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T3 T3 T3 T3
0 24 48 72 96 Hours
B
Ki67 Acinar
pH3 Acinar
Ki67+ cells/field
pH3+ cells/field
Cntl T3
C
Ki67 Acinar
Ki67+ cells/field
96 Hours
Cntl
400µg/Kg
80µg/Kg
16µg/Kg
3.2µg/Kg
D
PU.1
PU.1+ cells/field
Cntl T3
E
Hdac1
Hdac2
Hdac3
Hdac8
Hdac4
Hdac5
Hdac6
Hdac7
ΔΔCt
Cntl T3
Class I HDAC
Class II HDAC
F
HDAC activity
O.D.
Cntl T3
G
Ki67 Acinar
pH3 Acinar
Ki67+ cells/field
pH3+ cells/field
96 Hours
Cntl
T3
T3 + Ms
T3
T3 + MS
Ki67
pH3
96 Hours
H
Akt1
Akt2
Akt3
ΔΔCt
Cntl T3
I
Akt/α-Tubulin
pAkt/Akt
O.D. ratio
Cntl T3
Cntl
T3
pAkt (Ser473)
Akt
α-Tubulin
J
pAkt/Akt
O.D. ratio
96 Hours
Cntl
T3
T3 + MK
K
T3
T3 + MK
Ki67
pH3
96 Hours
Ki67 Acinar
pH3 Acinar
Ki67+ cells/field
pH3+ cells/field
96 Hours
Cntl
T3
T3 + MK

Figure 4

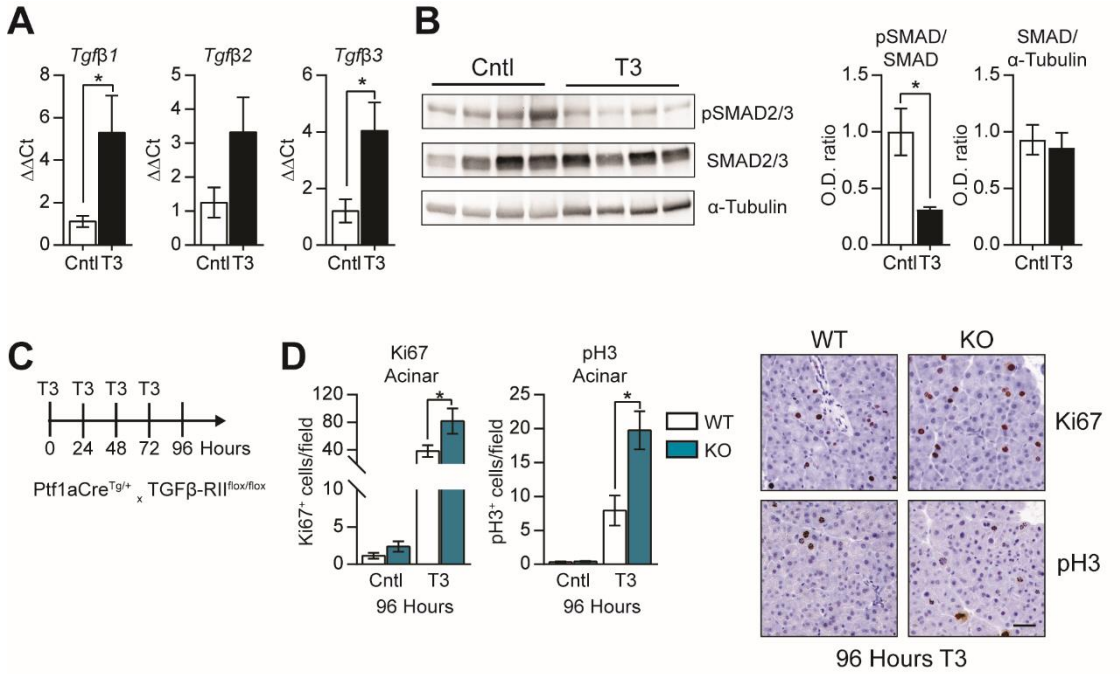

A
Tgfβ1
Tgfβ2
Tgfβ3
ΔΔCt
Cntl T3
B
Cntl
T3
pSMAD2/3
SMAD2/3
α-Tubulin
pSMAD/ SMAD
SMAD/ α-Tubulin
O.D. ratio
C
T3 T3 T3 T3
0 24 48 72 96 Hours
Ptf1aCre^Tg/+ x TGFβ-RII^flox/flox
D
Ki67 Acinar
pH3 Acinar
Ki67+ cells/field
pH3+ cells/field
WT
KO
96 Hours
WT
KO
Ki67
pH3
96 Hours T3

Figure 5

Supplementary figure legends

Figure S1. (A) Quantification of amylase and lipase activity in serum in untreated mice. **(B)** Quantification of Ki67 and pH3-positive acinar cells in untreated mice, one month after tamoxifen treatment. Right panels, representative microphotographs of stained cells. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure S2. (A) Quantification of acinar cells positive for the apoptotic marker cleaved caspase 3 96 hours after induction of pancreatitis. Right panel, representative microphotographs of stained cell **(B)** Quantification of acinar cells positive for the DNA damage marker pH2AX 96 hours after induction of pancreatitis Right panels, representative microphotograph of stained cell. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

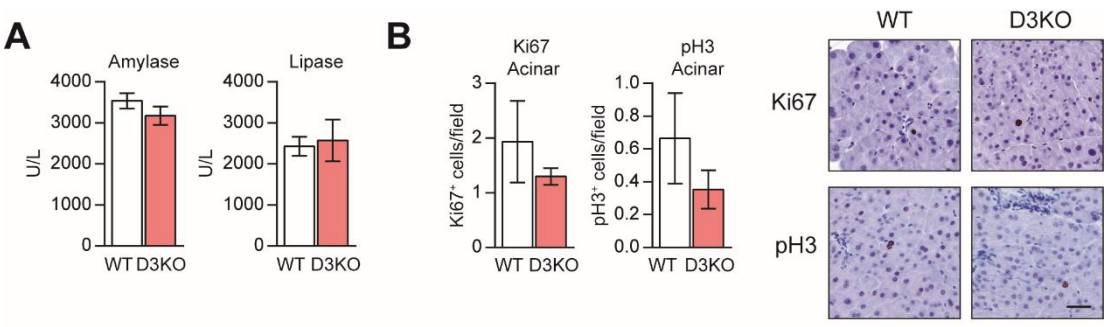
Figure S3. (A) qPCR of cyclin expression in the pancreas 96 hours after T3 administration. **(B)** Hematoxylin and Eosin (H&E) staining of pancreata 96 hours after T3 administration. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure S4. (A) Schematic representation of T3 treatment in the presence of the selective class I inhibitor MS-275 (MS). **(B)** Quantification of HDAC activity in the pancreas 96 hours after T3 treatment in the presence of MS. **(C)** qPCR of cyclin expression in the pancreas 96 hours after T3 treatment in the presence of MS. **(D)** qPCR of cell cycle inhibitor expression in the pancreas 96 hours after T3 treatment in the presence of MS. Results are average ± SEM (n=5), *P < 0.05.

Figure S5. (A) Schematic representation of T3 treatment in the presence of the selective Akt inhibitor MK-2206 (MK). **(B)** qPCR of cyclin expression in the pancreas 96 hours after T3 treatment in the presence of MK. **(C)** qPCR of cell cycle inhibitor expression in the pancreas 96 hours after T3 treatment in the presence of MK. Results are average ± SEM (n=5), *P < 0.05.

Figure S6. (A) qPCR of cyclin expression in the pancreas 96 hours after T3 treatment in wild type (WT) and TGFβ receptor II deficient (KO) mice. **(C)** qPCR of cell cycle inhibitor expression in the pancreas 96 hours after T3 treatment. Results are average ± SEM (n=5), *P < 0.05.

Figure S1



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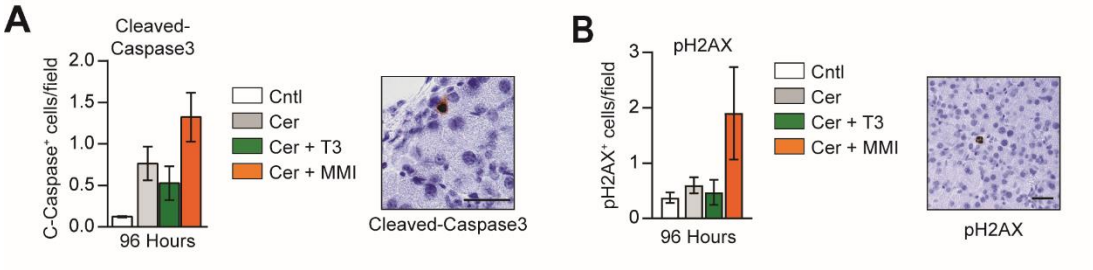

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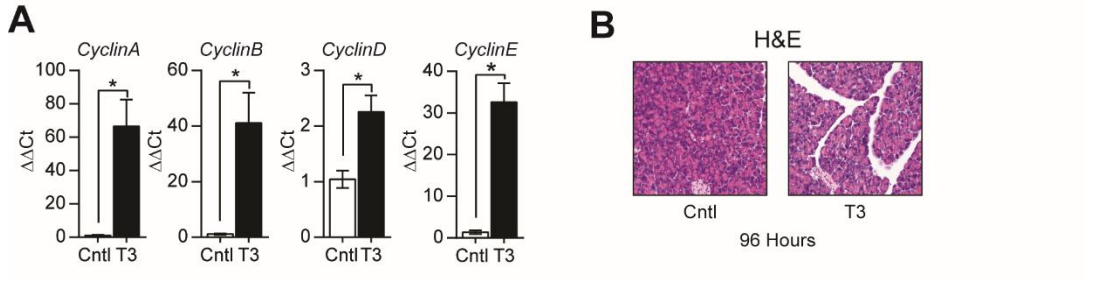

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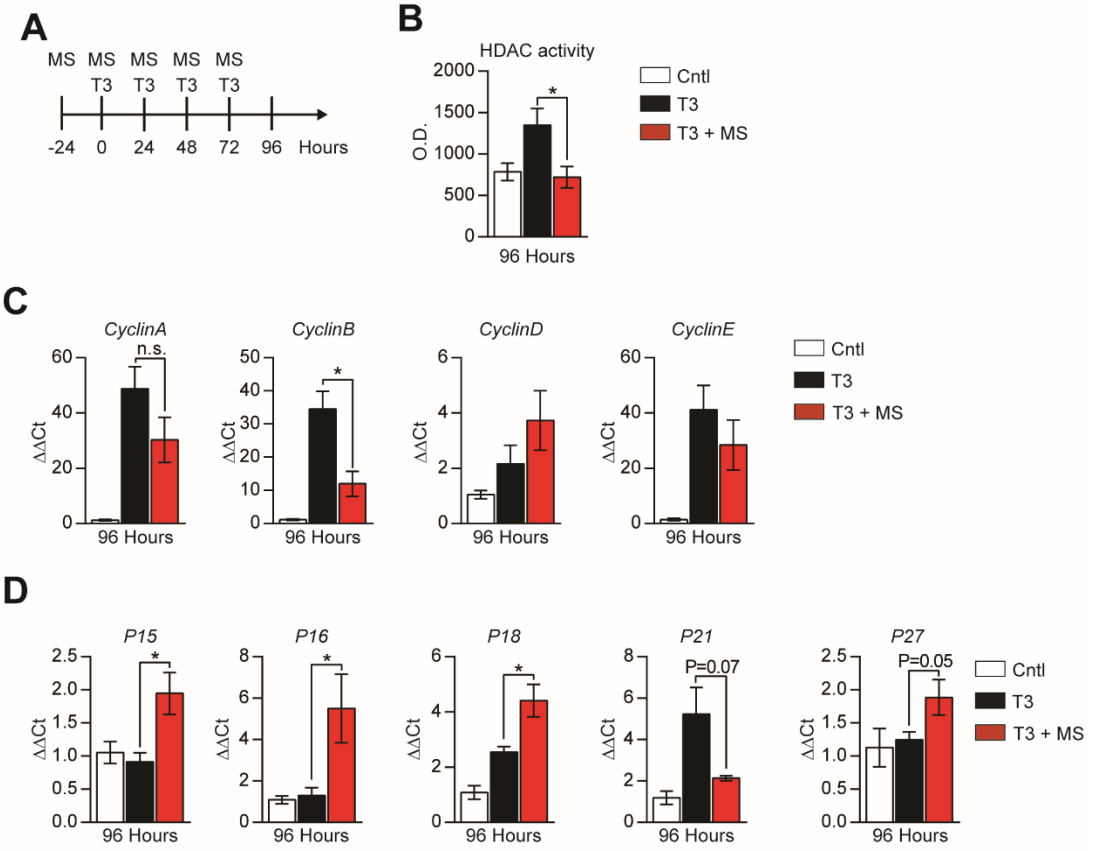

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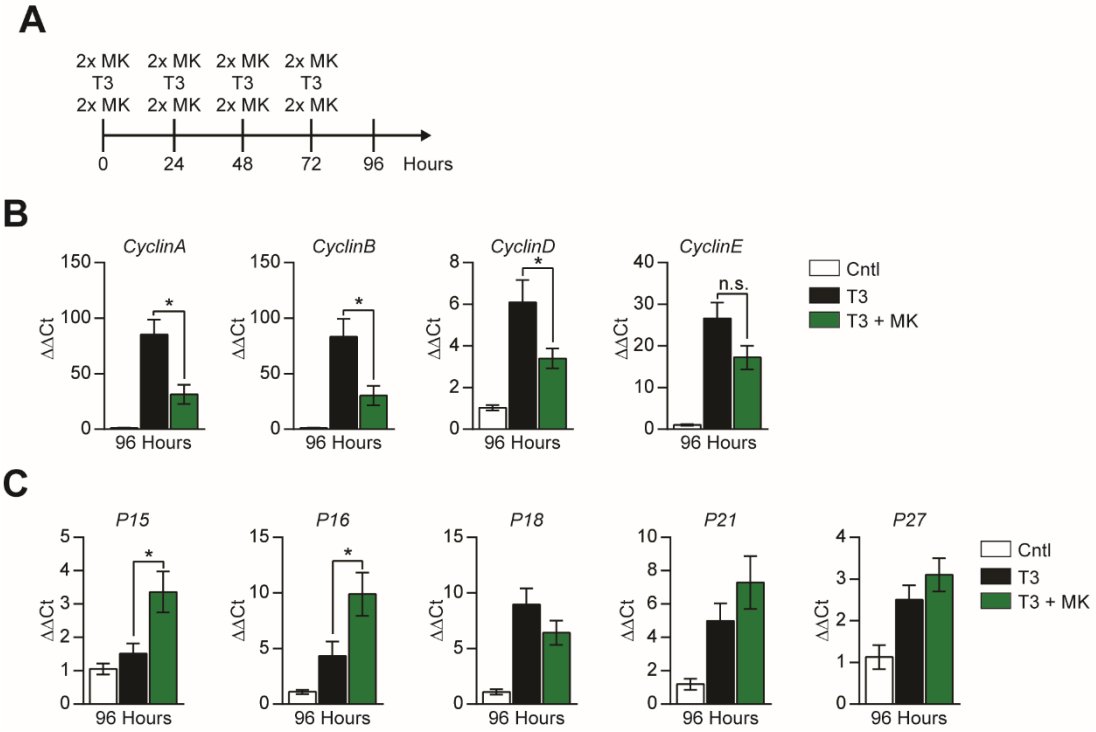

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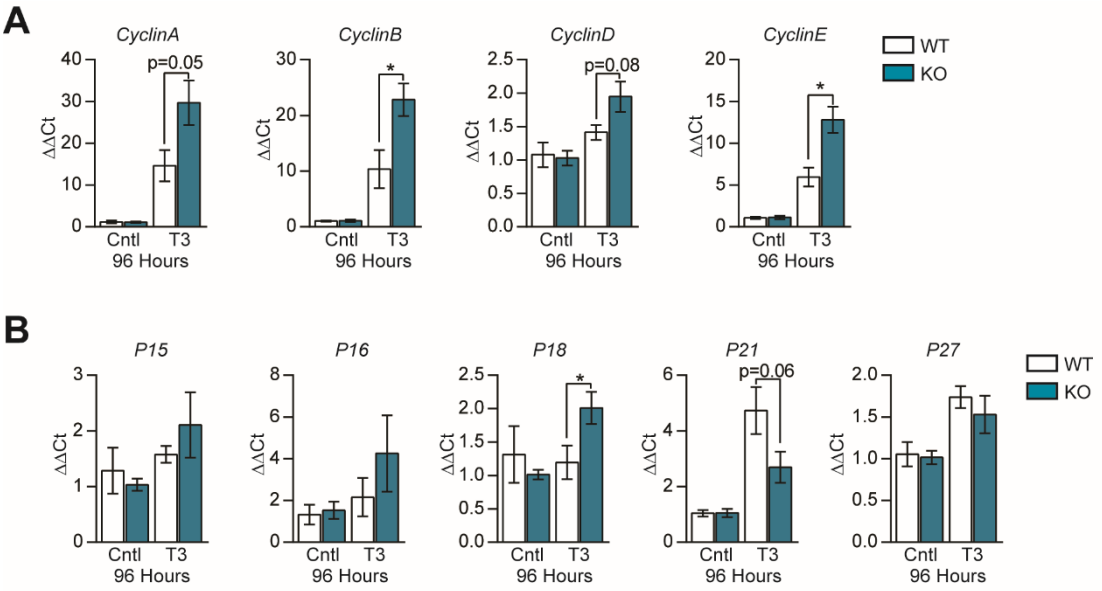

5. Manuscript B

Ibuprofen and diclofenac treatments reduce proliferation of pancreatic acinar cells upon inflammatory injury and mitogenic stimulation.

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Short title: Ibuprofen reduces acinar cell proliferation

Conflicts of interest: The authors disclose no conflicts.

Contribution: This paper represents part of my work completed during the third year of my PhD, I mostly contributed to this paper by characterizing the effect of Ibuprofen on acinar cell proliferation, upon T3 mitogenic stimulation.

Abstract

Background and Purpose Nonsteroidal anti-inflammatory drugs (NSAIDs) are administered to manage pain typically found in patients suffering from pancreatitis. NSAIDs also display anti-proliferative activity against cancer cells, however their effects in normal, untransformed cells is poorly understood. Here we evaluated whether NSAIDs inhibit proliferation of pancreatic acinar cells during the development of acute pancreatitis.

Experimental Approach The NSAIDs ibuprofen and diclofenac were administered to C57BL/6 mice after induction of pancreatitis with serial injection of cerulein. In addition, ibuprofen was administered concomitantly to 3,5,3-L-tri-iodothyronine (T3), which induces acinar cell proliferation in the absence of tissue inflammation. Development of pancreatic inflammation, acinar de-differentiation into metaplastic lesions and acinar proliferation were quantified by histochemical, biochemical and RT-PCR approaches.

Key Results Therapeutic ibuprofen treatment selectively reduced pancreatic infiltration of activated macrophages *in vivo*, M1 macrophage polarization and pro-inflammatory cytokine expression both *in vivo* and *in vitro*. Reduced macrophage activation was accompanied by reduced acinar de-differentiation into acinar-to-ductal metaplasia. Acinar proliferation was significantly impaired in the presence of ibuprofen and diclofenac, evidenced both at the level of proliferation markers and expression of cell cycle regulators. Ibuprofen treatment also reduced acinar cell proliferation induced upon mitogenic stimulation with T3, a treatment that does not elicit pancreatic inflammation.

Conclusions and Implications Our study provides evidence that the NSAIDs ibuprofen and diclofenac inhibit pancreatic acinar cell division. This suggests that prolonged treatment with these NSAIDs may negatively affect the extent of pancreatic regeneration and further studies are needed to confirm these findings in a clinical setting.

ABBREVIATIONS

NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclooxygenase; i.p.. intraperitoneal; T3, 3,5,3-L-tri-iodothyronine; ADM, acinar-to-ductal metaplasia.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are a broad family of compounds primarily used as analgesics to treat pains of different origin and to control inflammation. At the molecular level, NSAIDs exert these effects by inhibiting the activity of cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2) (Chavez and DeKorte, 2003), enzymes that catalyze the synthesis of prostaglandins and thromboxanes. In addition to the established and well exploited analgesic and anti-inflammatory effects of these compounds, numerous studies have demonstrated that NSAIDs are also effective in the prevention of many common cancers (Taketo, 1998, Vainio, 2001). This is particularly evident in the case of ibuprofen, the most commonly used over-the-counter NSAID (Bushra and Aslam, 2010). Specifically, this drug showed a superior effectiveness compared with other NSAIDs in suppressing proliferation and inducing apoptosis of human prostate cancer cells at clinically relevant concentrations (Andrews et al., 2002). Similar inhibition of proliferation exerted by ibuprofen, alone or in combination therapy, was also observed in the case of gastric (Bonelli et al., 2011), lung (Endo et al., 2014), colon (Greenspan et al., 2011, Reddy et al., 1992) and breast cancer, suggesting that ibuprofen may be useful in the chemoprevention of different malignancies (reviewed in (Piazza et al., 2010, Gurpinar et al., 2014).

While the anti-proliferative effect of ibuprofen is well documented in the case of cancer cells, the impact of this drug on the proliferation of normal cells in the absence of malignant transformation is poorly elucidated. Ibuprofen is amongst the therapeutic interventions administered to relief the pain characteristic for patients suffering from mild forms of acute pancreatitis, or, in the most severe cases of the disease, when patients are weaned off narcotic therapy. In addition, it is also used to manage chronic pancreatitis, where the permanent inflammation of the organ is associated with continuing pain (reviewed in (Banks et al., 2010)). NSAID treatment has also been shown prophylactic effects to prevent post-operative pancreatitis when given prior to endoscopic retrograde cholangiopancreatography (ERCP) (Murray et al., 2003, Sotoudehmanesh et al., 2007). Surprisingly, extensive studies to evaluate the efficacy of NSAID during acute pancreatitis are missing. A recent review of the available literature highlighted the fact that, while NSAIDs are able to control pain in acute pancreatitis patients, the use of NSAIDs is also associated with the risk for developing acute pancreatitis (reviewed in (Raffaele Pezzilli, 2010)). Thus, further clinical trials are needed to identify the optimal NSAID to be used in the management of pancreatitis.

In this study we evaluated whether the therapeutic administration of the NSAIDs ibuprofen and diclofenac affect the course of acute pancreatitis in terms of progression of inflammation and regeneration of the organ, using the most widespread murine model of the disease based on cerulein treatment. In addition, we investigated whether ibuprofen, administered in the absence of inflammatory insult, is able to directly inhibit mitogen-induced proliferation of pancreatic acinar cells.

METHODS

The drug/molecular target nomenclature conforms to the BJP's Concise Guide to Pharmacology (Alexander et al., 2015).

Animal experiments

All animal experiments were performed in accordance with Swiss Federal animal regulations and approved by the cantonal veterinary office of Zurich. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. Mice used in this study were adult 8–12 week old C57BL/6 mice in a weight range of 25-30 g (Envigo Laboratories, Horst, The Netherlands). Groups of 4-5 mice were kept in standard individually ventilated cages (IVCs) in a SPF facility. Only male mice were used in this study. Pancreatitis was induced via six intraperitoneal (i.p.) injections of 50 ▪g/kg cerulein, at hourly interval. In the "staggered" protocol, cerulein treatment was performed on three alternate days (Monday, Wednesday and Friday) and animals were harvested on the following Monday, seven days after the initial cerulein injection. In the "consecutive" protocol, cerulein treatment was performed on two consecutive days (Monday and Tuesday) and animals were harvested on the following Monday, seven days after the initial cerulein injection. Ibuprofen and diclofenac (Sigma) were injected twice daily, two hours apart, i.p. at 25 mg/kg and 10 mg/kg, respectively. Control animals for cerulein, ibuprofen and diclofenac treatments received vehicle (saline solution, 0.9% NaCl) injections. In the "staggered" protocol of pancreatitis, ibuprofen was administered for five days starting two hours after the second set of cerulein injections. In the "consecutive" protocol of pancreatitis, ibuprofen and diclofenac were administered for five days starting 24 hours after the second set of cerulein injections.

3,5,3-L-tri-iodothyronine (T3) was administered daily i.p. at 400 mg/kg. Stock solution of 2mg/mL T3 were prepared in 0.1M NaOH, pH 7.4 and freshly diluted in saline to the final concentration required for the *in vivo* experiments. Control animals received vehicle injections. Ibuprofen was injected twice daily, i.p. at 25 mg/kg, one hour before and one hour after T3 injections. Schematic representations of the different study groups are depicted in the relevant figures.

Animal harvest was performed under isoflurane anesthesia. Groups of 5 animals were tested for each experiment and time point. Animals were assigned randomly to different experimental groups for all in vivo studies. Data collection and evaluation of all in vivo and in vitro experiments were performed blindly of the group identity.

Mammalian cell cultures

The RAW264.7 macrophage cell line was maintained in Dulbecco's Modified Eagle medium (DMEM)+GlutaMAX supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were pre-incubated with 800 ▪M ibuprofen for 30 minutes and stimulated with 10 ng/mL LPS for 16 hours in the presence or absence of ibuprofen.

The AR42J acinar cell line was maintained in F12K medium supplemented with 20% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. For proliferation experiments, cells were seeded in 96 well-plates and incubated with different concentrations of ibuprofen for 72 hours.

Cell number, cell viability and cell diameter were determined using an automated cell counter (NucleoCounter® NC-200™, Chemometec, Allerod, Denmark).

Transcript analysis

Total RNA was extracted from pancreatic tissue, as described previously (Graf et al., 2002), or cell lines and reverse-transcribed with qScript™ cDNA SuperMix (Quanta Biosciences). Gene expression was measured by real-time PCR on a 7500 Fast Real-Time PCR System (Applied Biosystems) using Taqman probes (Applied Biosystems). Transcript levels were normalized using 18S RNA as a reference and expressed as ‥Ct relative to the value of control samples.

Immunohistochemistry

Pancreas specimens were fixed overnight in neutral buffered formalin, dehydrated through a series of graded ethanol baths and embedded in paraffin. For histological analyses, 3 ‧m tissue sections were deparaffinized in xylol and rehydrated in graded ethanol. Following antigen retrieval in citrate buffer and neutralization of endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol, sections were incubated with protein block solution (Dako), primary and secondary antibodies and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Vectastain® ABC HRP Kit, Vector Laboratories, Peterborough, UK), according to the manufacturer's protocol. Primary antibodies used in this study were: rabbit anti-Ki67 (#ab16667, Abcam, Cambridge, UK); rabbit anti-phospho-histone 3 (#2066052, Millipore, MA, USA); rabbit anti-amylase (#A8273-1VL, Sigma-Aldrich, Buchs, Switzerland); rabbit anti-PU.1 (#2266, Cell Signaling Technologies, Danvers, MA); rabbit anti-‧H2A.X (Ser139) (#9718, Cell Signaling Technologies, Danvers, MA); rat anti-F4/80 (#T-2006 BMA Biomedicals, Switzerland); rabbit anti-CD3 (#A 0452, Dako); rabbit anti-cleaved Caspase-3 (Asp175) (#9661, Cell Signaling Technologies, Danvers, MA); mouse anti-YM1/Chitinase 3-like 3 (#AF2446, R&D Systems, MN, USA); rabbit anti-IRF-5 (#10547-1-AP, Proteintech, Manchester, UK).

Secondary antibodies used in this study were biotinylated goat anti-rabbit IgG (H + L), included in the Vectastain® ABC HRP Kit. Nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI).

Detection of DNA fragmentation in apoptotic cells was performed with a TUNEL assay using an ApopTag peroxidase Kit (MP Biomedicals, Illkirch, France), according to the manufacturer's instructions.

For quantitative analysis of ADM, paraffin-embedded pancreas specimens were immunostained for amylase, slides were scanned with a NDP NanoZoomer Digital Pathology Slide Scanner (Hamamatsu) and analyzed for ADM lesions in a blinded fashion. ADM present in the entire pancreas slide of 5 mice from each treatment condition were quantified by manual counting. ADM were identified according to: i) loss of amylase content, ii) structural re-organization into tubular complexes, iii) stromal reaction characterized by presence of cell infiltrates. The area occupied by ADM was expressed as percentage of total pancreatic area present in each slide.

The amount of amylase and F4/80 expressing macrophages present in the tissue was determined by densitometric quantification in at least 10 random high-power fields per slide using the Cell^P analysis software (Olympus). Positive area was expressed as percentage of total pancreatic area present in each power field. Pancreatic ducts and vessels were excluded from the analysis.

Microscopy analyses were performed on a wide-field Nikon Eclipse Ti (Amsterdam, The Netherlands). Quantification of labelled cells was performed in at least 10 randomly selected high-power fields (×200)

per slide using the NIS Elements BR Analysis and Cell^P analysis software.

Data and analysis

The data are expressed as means ± SEM. The statistical significance of differences in the means of experimental groups was determined using an unpaired, two-tailed Student's *t* test (GraphPad Prism 4.0c; GraphPad Software, Inc.) and a probability value <0.05 was considered statistically significant. Data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 (Alexander et al., 2015).

RESULTS

Ibuprofen treatment reduces pancreatic recruitment of activated macrophages following induction of acute pancreatitis.

To assess the effect of ibuprofen during the progression of pancreatitis, we administered the drug in a therapeutic manner starting two days after induction of the disease via cerulein injections (regimen scheme depicted in Fig. 1A). In this experimental setting, the initial acinar injury is comparable in the control and ibuprofen-treated groups. Ibuprofen was applied at a dose similar to the range used in human therapy (Janssen and Venema, 1985), and which was demonstrated to inhibit prostaglandin production and pain perception in mice (Salama et al., 2016). Animals were harvested seven days after the first cerulein injection. Following induction of pancreatitis, ibuprofen-treated mice displayed histological alterations of pancreatic parenchyma similar to vehicle-treated mice (Fig. 1B) and a comparable pattern of weight loss (Fig. S1). We then investigated whether ibuprofen reduced the infiltration of inflammatory cells. The total number of leukocytes was only slightly lowered in the presence of the drug (Fig. 1C). However, ibuprofen reduced the level of macrophages recruited in the pancreas, tested both via F4/80 immunostaining, detecting activated macrophages, and gene expression levels (Fig. 1D, F). On the contrary, the number of CD3-positive T cells was comparable in control and treated samples (Fig. 1D, F). These data suggest that ibuprofen targets the infiltration of selected sub-populations of inflammatory cells. Concomitant with reduced macrophage infiltration, ibuprofen treatment also reduced the expression of selected cytokines, chemokines and adhesion molecules normally up-regulated in the pancreas following induction of pancreatitis (Fig. 1G). To test whether ibuprofen directly inhibits macrophage activation and cytokine expression, we treated RAW 264.7 macrophages with the drug *in vitro* and quantified their activation upon LPS stimulation. RAW cells responded to 16 hours of LPS treatment, as evidenced by a change in cell shape to a flattened, pancake-like morphology (McWhorter et al., 2013) (Fig. 2A), increased cell diameter (Fig. 2B) and reduced proliferation (Fig. 2C). Ibuprofen did not alter these parameters, however, similarly to what was observed *in vivo*, it reduced the expression of selected cytokines in LPS-treated macrophages (Fig. 2D). This suggests that ibuprofen directly counteracts the functional activation of macrophages.

Ibuprofen treatment reduces ADM formation following induction of acute pancreatitis.

We then further analyzed the inhibitory effect of ibuprofen on macrophages, as these cells play an essential role in the context of pancreatitis. Indeed, macrophages orchestrate both the initiation and the resolution of inflammation (reviewed in (Shrivastava and Bhatia, 2010)). In addition, M1 polarized macrophages directly influence the status of acinar cells, as they can initiate and drive the transient trans-differentiation of acinar cells into acinar-to-ductal metaplasia (ADM), which occurs during the regenerative phase of pancreatitis (Liou et al., 2013, Liou and Storz, 2015). Specifically, F4/80-positive activated macrophages are abundantly found associated with ADM lesions, where M1 macrophages are more abundant than M2 macrophages (Fig. 3A). Ibuprofen decreased the expression of M1 macrophage markers (Cd40, iNos2), without altering the levels of M2 macrophage markers (Arg1, Clec10) (Fig. 3B). Amongst the cytokines secreted by M1 macrophages, TNF and RANTES/CCL5 have been previously identified as the major inducers of acinar cell reprogramming into ADM (Liou et al., 2013). We found that ibuprofen treatment limited the expression of both cytokines in the pancreas upon induction of pancreatitis (Fig. 3C).

Finally, we analyzed whether the reduced amount of M1 macrophages and ADM-promoting cytokines observed in the pancreas following ibuprofen treatment was associated with and reduced ADM formation. Ibuprofen-treated mice showed a decreased trend of mature ADM lesions, morphologically defined by loss of amylase expression, structural re-organization into tubular complexes and stromal reaction with robust cell infiltration (Fig. 3D, E), and decreased trend, albeit not significant, of the ADM marker CK19 expression (Fig. S2). In addition, densitometric quantification of amylase levels, which allows the determination of tissue with intact acinar cells, showed larger area of amylase-expressing acinar cells devoid of ADM upon ibuprofen treatment (Fig. 3F). Collectively, these data indicate that inhibition of macrophage recruitment/function mediated by ibuprofen treatment is likely to limit acinar de-differentiation into metaplastic lesions following induction of pancreatitis.

Ibuprofen treatment reduces acinar cell proliferation following induction of acute pancreatitis.

Adult pancreatic acinar cells have the ability to initiate a proliferation program following pancreatitis to regenerate damaged tissue. As ibuprofen limits the proliferation of different cancer cell types, we tested whether the drug also reduced the proliferation of untransformed acinar cells. Ibuprofen treatment decreased the number of acinar cells expressing the proliferation markers Ki67 and phosphor-histone 3 (pH3) (Fig 4A). Expression of both early and late cyclins was also reduced (Fig. 4B). Reduced acinar proliferation was not caused by up-regulation of cyclin-dependent kinase inhibitors (CDKi). Indeed, ibuprofen-treated pancreata showed a general decrease in CDKi and p53 expression (Fig. 4C). Ibuprofen exerts its anti-proliferative actions on several immortalized cell lines, including microglia (Elsisi et al., 2005), glioma (Gomes and Colquhoun, 2012) and gastric cancer cells (Bonelli et al., 2011), through induction of apoptosis. Thus we tested whether apoptotic cell death was induced in acinar cells upon treatment with the drug. Quantification of cleaved caspase 3 (Fig. 4D) and DNA fragmentation by TUNEL assay (Fig. S3A) showed that ibuprofen did not increase apoptosis of acinar cells in the context of pancreatitis. Similarly, ibuprofen treatment

did not increase the levels of DNA damage in acinar cells (Fig. S3B) or the expression of heat shock protein 72 and Bcl2 (Fig. 4E), which exert a protective effect against cell death in the pancreas (Saluja and Dudeja, 2008). Collectively, these results showed that ibuprofen administration reduced acinar cell proliferation in the context of pancreatitis without engaging a cell death program previously reported in ibuprofen-treated cancer cells.

Finally, we tested whether ibuprofen was effective in reducing acinar proliferation when administered after the completion of cerulein treatment. To this aim, cerulein was applied on two consecutive days followed by five days of ibuprofen (scheme in Fig. 5A). Ibuprofen reduced proliferation of acinar and interstitial cells also in this protocol of acute pancreatitis (Fig. 5B, C). In addition, similar inhibition was achieved by the non-selective COX inhibitor diclofenac (Fig. 5A-C), suggesting that different NSAIDs share the ability to interfere with acinar cell proliferation.

Ibuprofen treatment reduces acinar cell proliferation upon T3-induced mitogenic stimulation.

While our results point out an anti-proliferative effect of ibuprofen in the context of pancreatitis, the altered inflammatory milieu observed in the presence of the drug may constitute a confounding factor that prevents the direct assessment of the inhibitory properties of ibuprofen in acinar cells. Thus, we tested the effect of ibuprofen on acinar cell proliferation in an experimental model independent from inflammation. To this aim, mice were treated for four days with the thyroid hormone 3,5,3-L-tri-iodothyronine (T3), which acts as a mitogen for pancreatic acinar cells (Kowalik et al., 2010, Ledda-Columbano et al., 2005), concomitantly with ibuprofen administration (scheme depicted in Fig. 6A). As expected, T3 did not induce leukocyte recruitment in the pancreas (Fig. 6B) or up-regulation of COX enzymes (Fig. 6C). Importantly, ibuprofen treatment did not change basal leukocyte levels (Fig. 6B), but selectively decreased proliferation of acinar cells without affecting interstitial cells (Fig. 6D). Similar to what was observed in the context of pancreatitis, reduction of acinar cell proliferation was not associated with increased apoptotic cell death of acinar cells (Fig. S4). In a further experiment, we tested whether ibuprofen delayed acinar cell division resulting in a long-lasting proliferation index. Four days after the last T3 administration, acinar proliferation was reduced to near base-line levels in both groups, suggesting that ibuprofen-treated mice responded to T3 withdrawal without residual proliferation activity at the time point analyzed (Fig. 6E, F).

Collectively, these results show that ibuprofen is a *bona fide* inhibitor of acinar cell proliferation induced by T3 stimulation.

Finally, ibuprofen was also tested *in vitro* on the pancreatic acinar cell line AR42J. Despite their tumor origin, these cells are quite unique in retaining morphological and functional characteristics typical of adult acinar cells, albeit harbouring an unrestrained proliferative ability. Similar to what observed *in vivo*, *in vitro* treatment with clinically relevant concentrations of ibuprofen (Andrews et al., 2002) reduced the proliferation of AR42J cells in a dose-dependent manner (Fig. S5).

DISCUSSION

Current treatment of pancreatitis primarily involves supportive therapy, which includes pain relief and

prevention of infection (Paisley and Kinsella, 2014, Bang et al., 2008). In this context, NSAIDs are frequently used in virtue of their analgesic as well as anti-inflammatory effects. However, preclinical studies demonstrated that NSAIDs are also effective in inhibiting the proliferation of a wide range of cancer cells *in vitro*, either alone or in combination with other cancer therapies. This is further supported by clinical trials demonstrating that long term use of NSAIDs, including ibuprofen, significantly reduces the risk of colorectal, breast, lung, prostate and gastric cancer and inhibits proliferation of cancer cells, including glioma, neuroblastoma, bladder cancer cells (Ulrich et al., 2006, Johnson et al., 2010, Baron and Sandler, 2000, Leidgens et al., 2015, Ikegaki et al., 2014, Chai et al., 2015, Fajardo and Piazza, 2015), thus increasing the interest for a novel therapeutic application of these drugs. However, this observation raises the question whether the inhibition of cell proliferation mediated by NSAIDs is also observed in non-malignant cells, with the consequence that the drug treatment results in delayed regeneration of tissues following injury.

In the present study we investigated the effect of the NSAIDs ibuprofen and diclofenac in the regeneration of pancreatic tissue *in vivo* following induction of pancreatitis. Our results showed that administration of these NSAIDs significantly reduced the proliferation of acinar cells. Given the anti-inflammatory properties of ibuprofen, we then investigated whether reduced acinar proliferation was derived from a reduced inflammatory response. We did not observe a general decrease of pan-leukocyte infiltration in the pancreas upon ibuprofen treatment. However, macrophage levels and pro-inflammatory cytokine/chemokine expression were reduced, indicating that the drug affected the recruitment of selected leukocyte populations and likely their activation. This was further confirmed in *in vitro* experiments where ibuprofen treatment hampered LPS-induced activation and cytokine expression in macrophages. Reduced inflammation may reduce inflammation-dependent damage of acinar cells and consequently the need of acinar cell proliferation. However, this direct correlation was not observed, as DNA damage and apoptosis were unchanged in acinar cells in the presence of the drug.

In this context, it is worth mentioning that expression of the anti-apoptotic marker Bcl2 was reduced during pancreatitis upon ibuprofen treatment. While the exact mechanisms of this lower expression are not completely defined, it is likely that this phenotype is a consequence of reduced inflammation observed following NSAID administration, as inflammatory mediators have been reported to regulate Bcl2 expression in various types of cells (Minshall et al., 1997, Pugazhenthi et al., 1999). However, another intriguing possibility is that lower Bcl2 levels reflect the reduced proliferation of acinar cells. Indeed, the cellular role of Bcl2 is not restricted to regulating apoptosis but it also influences cell proliferation by restraining entry into the cell cycle and promoting quiescence (reviewed in (Cory et al., 2003)). Thus, reduced acinar proliferation in the presence of ibuprofen may result in reduced Bcls2 induction to limit its negative control of cell division.

In addition, ibuprofen treatment reduced acinar cell proliferation also in an inflammation-independent setting, suggesting that the anti-inflammatory and anti-proliferative effects are two independent outcomes of ibuprofen treatment. On the other hand, the anti-inflammatory effect of ibuprofen is likely to impact on the formation of metaplastic lesions triggered by induction of pancreatitis. In this respect, ibuprofen treatment limited pancreatic infiltration of M1 macrophages. The critical role of this cell type

and their secreted cytokines in the trans-differentiation of acinar cells into ADM was shown recently (Liou et al., 2013). Amongst numerous cytokines produced by macrophages, only TNF and RANTES/CCL5 were able to drive acinar cell trans-differentiation through activation of NF-kB activity and NF-kB-induced target genes. Importantly, expression of these cytokines was also reduced in our ibuprofen-treated pancreata, thus likely explaining the reduced trend of ADM formation observed upon induction of pancreatitis.

The striking inhibition of acinar proliferation we observed upon ibuprofen and diclofenac treatment raises the question on the identity of molecular mechanisms underlining the phenotype. As the drugs target cyclooxigenases (COXs) and consequently reduces prostaglandin (PG) synthesis (Rome and Lands, 1975), one possibility is that PGs play a direct role in promoting cell proliferation. In support of this hypothesis, elevated COX2 activity and PGs production have been found in various hyper-proliferating cancer cells. Importantly, overexpression of this enzyme or exogenous administration of PGEs resulted in increased proliferation of different cancer cells (Sheng et al., 2001, Wang and Dubois, 2010, Menter and Dubois, 2012, Gomes and Colquhoun, 2012, Gu et al., 2008), indicating that COX2 activity and PGE synthesis are able to drive the cellular replicative program. In addition, signaling pathways which are often mutated and activated during cancer development, including Wnt and Ras pathways, have been shown to up-regulate COX2 expression (Araki et al., 2003), thus directly implicating COX2 activity as a key factor promoting tumorigenesis and cancer progression.

However, the reduced acinar cell proliferation observed following ibuprofen treatment is likely to be, at least partially, independent from COX2 activity. The key approach to answer this question was provided by our analysis of T3-induced acinar proliferation, an experimental setting that does not trigger the development of an inflammatory response. Indeed, we showed that COX2 was not expressed during the pronounced proliferation of acinar cells induced by T3 administration. Nevertheless, ibuprofen treatment reduced T3-induced acinar proliferation. In addition, we showed previously that pancreatitis is characterized by increased pancreatic expression of COX2 but acinar proliferation does not robustly decrease upon either genetic ablation or selective pharmacological inhibition of COX2 (Silva et al., 2011).

Collectively, these data indicate that ibuprofen limits acinar proliferation independently from its anti-inflammatory effect and that this inhibition of proliferation is likely mediated by COX2-independent mechanisms targeted by ibuprofen. In this context, COX-independent effects have been evoked also to explain the effectiveness of NSAIDs to inhibit cancer cell proliferation (reviewed in (Matos and Jordan, 2015)). Accumulating evidence that supports this hypothesis includes the fact that i) the anti-tumor effects of NSAIDs are typically seen at concentrations higher than those required to inhibit PGE synthesis (Tegeder et al., 2001, Grosch et al., 2006) and ii) NSAIDs still have antineoplastic effects when used against COX1 and COX2-deficient cells (Zhang et al., 1999).

One concept that emerges from the numerous reports describing the anti-proliferative actions of ibuprofen and other COX inhibitors in cancer cells is that the drug treatment induces cellular death through apoptosis. While this effect is undoubtedly important for the potential of NSAIDs as anti-cancer therapy, induction of cell death may not be a common outcome of ibuprofen administration to normal untransformed

cells. In fact, we did not detect increased apoptosis in the presence of ibuprofen in mice treated with the drug alone, following induction of pancreatitis or upon mitogenic stimulation with T3. Thus, it is likely that ibuprofen reduced acinar proliferation mainly via control of cell cycle. In support of this hypothesis, a very interesting study cross-examining the effect of ibuprofen in different eukaryotic model organisms, showed that the drug delays cell cycle progression through the G1 phase, in the absence of cancer-related pathologies. Importantly, this led to increased lifespan conserved in multiple species (He et al., 2014), even in yeast cells that are devoid of COX enzymes (Simmons et al., 2004). Further studies also demonstrated that ibuprofen reduced the proliferation of non-cancer cells, including endothelial and human coronary artery smooth muscle cells (Wiktorowska-Owczarek et al., 2015, Dannoura et al., 2014),

Conclusion

Modulation of the immune system has been proposed as a valid therapeutic strategy to mitigate the inflammatory response and consequently the severity of pancreatitis. Our work raised a caveat regarding the use of the commonly prescribed anti-inflammatory agents ibuprofen and diclofenac as it revealed that the drugs exert a significant anti-proliferative effect in acinar cells, as demonstrated in both inflammatory situation following induction of pancreatitis and non-inflammatory situation upon T3-induced mitogenic stimulation. Given that COX2 inhibitors have been reported to impair regeneration in a murine model of skin wound (Gourevitch et al., 2014), bone fracture healing (Simon and O'Connor, 2007), tendon healing (Connizzo et al., 2014), further studies are warranted to assess whether the anti-proliferative effect of these NSAIDs detected in acinar cells is detrimental for regeneration of the injured pancreas. In this regard, special interest should be focused not only on long term inflammatory damage of the organ in the context of chronic pancreatitis, but also on regeneration from tissue loss following pancreatectomy.

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Conflict of interest

The authors declare no conflicts of interest.

Ethics approval Animal experiments were approved by the cantonal veterinary office of Zurich in accordance with Swiss Federal animal regulations.

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Figure legends

Figure 1. Ibuprofen treatment reduces macrophage infiltration following acute pancreatitis. (**A**) Schematic representation of ibuprofen treatment using the "staggered" protocol of cerulein-induced acute pancreatitis. Light grey boxes represent six intraperitoneal (i.p.) injections of 50 μg/kg cerulein (Cer) administered hourly on alternate days. Dark grey boxes represent two i.p. injections of 25 mg/kg ibuprofen (Ibu) administered daily four hours apart. Black triangle indicates the time of animal harvest, counting from the first cerulein injection. (**B**) Haematoxylin and Eosin (H&E) staining of pancreata after the indicated treatments. (**C**) Quantification of leukocytes, positive for the pan-leukocytes PU.1, infiltrating the pancreas. Right panel, representative microphotograph of stained cells. (**D**) Quantification of F4/80-positive activated macrophages infiltrating the pancreas. Right panel, representative microphotograph of stained cells. (**E**) Quantification of CD3-positive T-cells infiltrating the pancreas. Right panel, representative microphotograph of stained cells. (**F**) qPCR of F4/80 and Cd3 expression. (**G**) qPCR of inflammatory cytokines, chemokine and adhesion molecule expression. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 2. Ibuprofen treatment reduces cytokine expression in LPS-activated macrophages. (**A**) Microphotograph showing morphological alteration and flattened shape (arrows) in RAW264.7 macrophages upon activation with 10 ng/mL LPS for 16h in the presence of 800 μM ibuprofen. (**B**) Cell diameter of LPS-treated RAW264.7 macrophages in the presence of ibuprofen. (**C**) Quantification of live and dead RAW264.7 macrophages treated

with LSP in the presence of ibuprofen. (**D**) qPCR of pro-inflammatory markers in RAW264.7 macrophages treated with LSP in the presence of ibuprofen. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 3. Ibuprofen treatment alters macrophage polarization and reduces ADM formation following acute pancreatitis. (**A**) Staining of F4/80-positive activated macrophages and M1, M2 polarized macrophages in ADM lesions (asterisks). (**B**) qPCR of M1 and M2 macrophage markers in pancreata. (**C**) qPCR of ADM-promoting pro-inflammatory cytokines. (**D**) Staining of amylase revealing intact acinar cells (brown areas) and ADM lesions with loss of amylase (asterisks). (**E**) Quantification of ADM areas following amylase staining, expressed as percentage of total area. (**F**) Quantification of intact acinar cell areas following amylase staining, expressed as percentage of total area. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 4. Ibuprofen treatment reduces acinar cell proliferation following acute pancreatitis. (**A**) Quantification of proliferating acinar cells upon staining with the general proliferation marker Ki67 and with the mitosis-specific marker pH3. Right panels, representative microphotographs of stained cells. qPCR of cyclins (**B**) and cell cycle inhibitors (**C**) in pancreata after induction of pancreatitis. (**D**) Quantification of cleaved caspase 3 (CC3)-positive apoptotic acinar cells after induction of pancreatitis. Right panel, representative microphotograph of stained cells. (**E**) qPCR of Hsp72 and Bcl2 expression in pancreata. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 μM.

Figure 5. Ibuprofen and diclofenac treatments reduce acinar cell proliferation in a second model of acute pancreatitis. (**A**) Schematic representation of NSAID treatment using the "consecutive" protocol of cerulein-induced acute pancreatitis. Light grey boxes represent six intraperitoneal (i.p.) injections of 50 μg/kg cerulein (Cer) administered hourly on two consecutive days. Dark grey boxes represent two i.p. injections of 25 mg/kg ibuprofen (Ibu) administered daily four hours apart. Black boxes represent two i.p. injections of 10 mg/kg diclofenac (Dic) administered daily four hours apart. Black triangle indicates the time of animal harvest, counting from the first cerulein injection. (**B**) Quantification of proliferating acinar cells upon staining with the general proliferation marker Ki67 and with the mitosis-specific marker pH3. (**C**) Quantification of proliferating interstitial cells upon staining with the general proliferation marker Ki67 and with the mitosis-specific marker pH3. Right panels, representative microphotographs of stained cells. Results are average ± SEM (n=5), *P < 0.05.

Figure 6. Ibuprofen treatment reduces acinar cell proliferation following mitogenic stimulation. (**A**) Schematic representation of ibuprofen treatment during stimulation with 3,5,3-L-tri-iodothyronine (T3). Light grey boxes represent daily intraperitoneal (i.p.) injections of 400 mg/kg T3. Dark grey boxes represent two i.p. injections of 25 mg/kg ibuprofen (Ibu) administered daily four hours apart. Black triangle indicates the time of animal harvest, counting from the first T3 injection. (**B**) Quantification of PU.1-positive pan-leukocytes infiltrating the pancreas. (**C**) qPCR of Cox1,2 expression in pancreata. (**D**) Quantification of proliferating acinar and interstitial cells upon staining with the general proliferation marker Ki67 and with the mitosis-specific marker pH3. (**E**) Schematic representation of prolonged ibuprofen treatment after stimulation with 3,5,3-L-tri-iodothyronine (T3). Symbols are as in (A). (**F**) Quantification of proliferating acinar cells upon staining with the general proliferation marker Ki67 and with the mitosis-specific marker pH3. Results are average ± SEM (n=5), *P < 0.05.

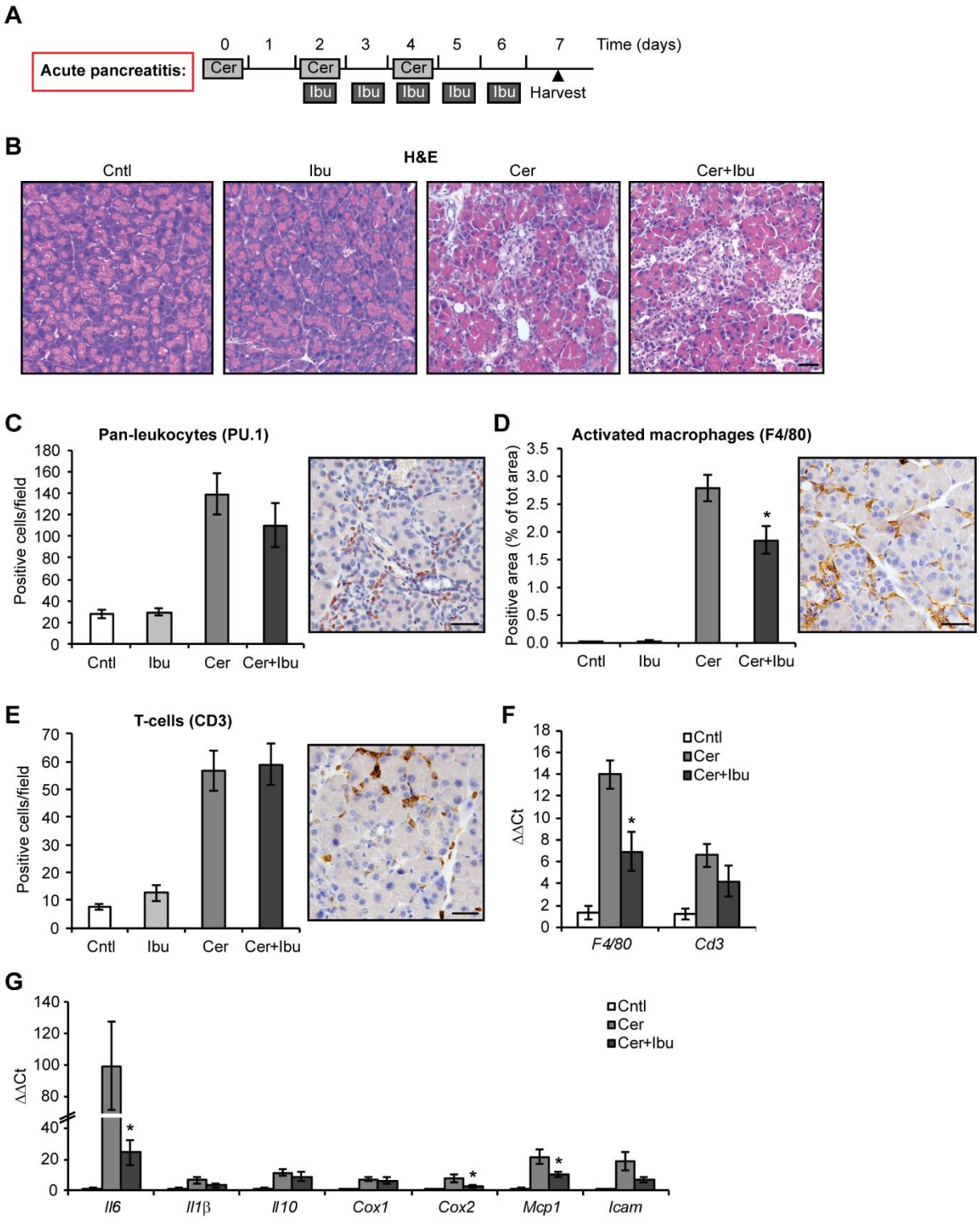

A
Acute pancreatitis:
0 1 2 3 4 5 6 7 Time (days)
Cer
Ibu
Harvest
B
H&E
Cntl
Ibu
Cer
Cer+Ibu
C
Pan-leukocytes (PU.1)
Positive cells/field
D
Activated macrophages (F4/80)
Positive area (% of tot area)
E
T-cells (CD3)
Positive cells/field
F
ΔΔCt
F4/80
Cd3
G
ΔΔCt
Il6
Il1β
Il10
Cox1
Cox2
Mcp1
Icam

Figure 1

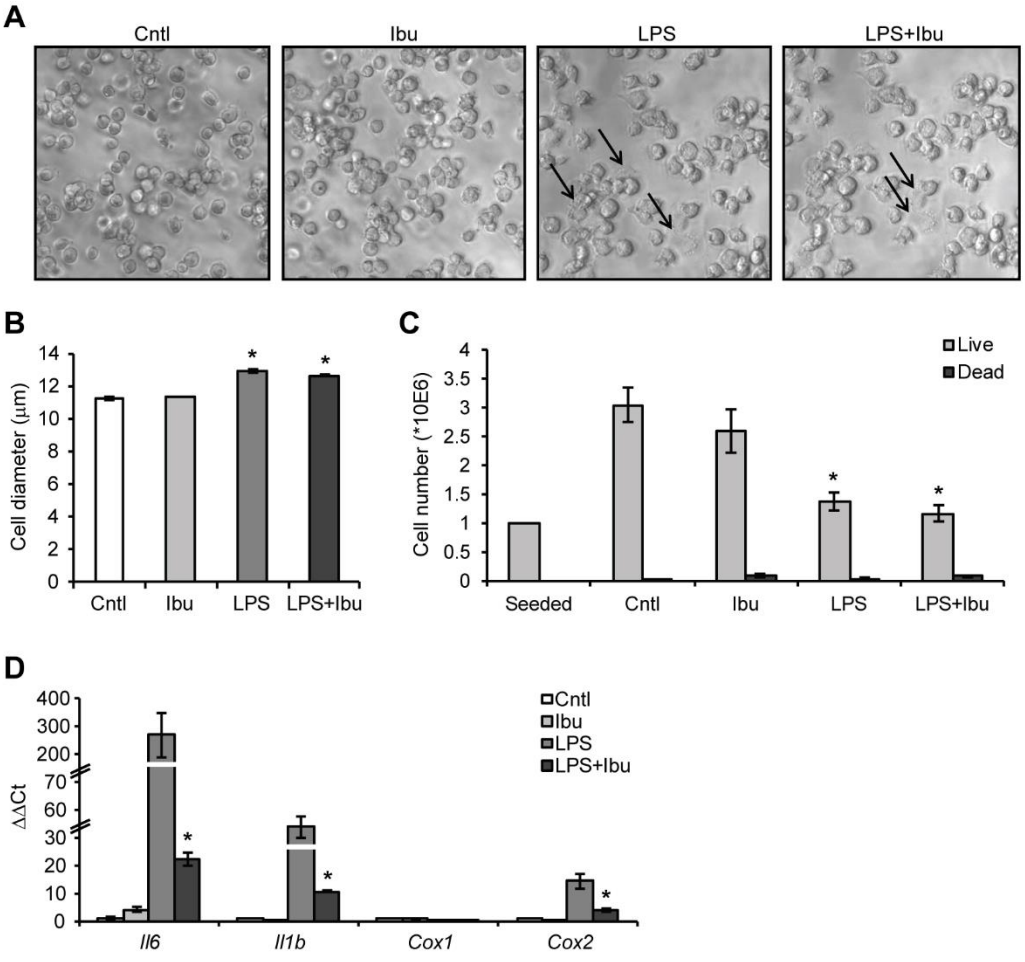

A
Cntl
Ibu
LPS
LPS+Ibu
B
Cell diameter (µm)
Cntl
Ibu
LPS
LPS+Ibu
C
Live
Dead
Cell number (*10E6)
Seeded
Cntl
Ibu
LPS
LPS+Ibu
D
Cntl
Ibu
LPS
LPS+Ibu
ΔΔCt
Il6
Il1b
Cox1
Cox2

Figure 2

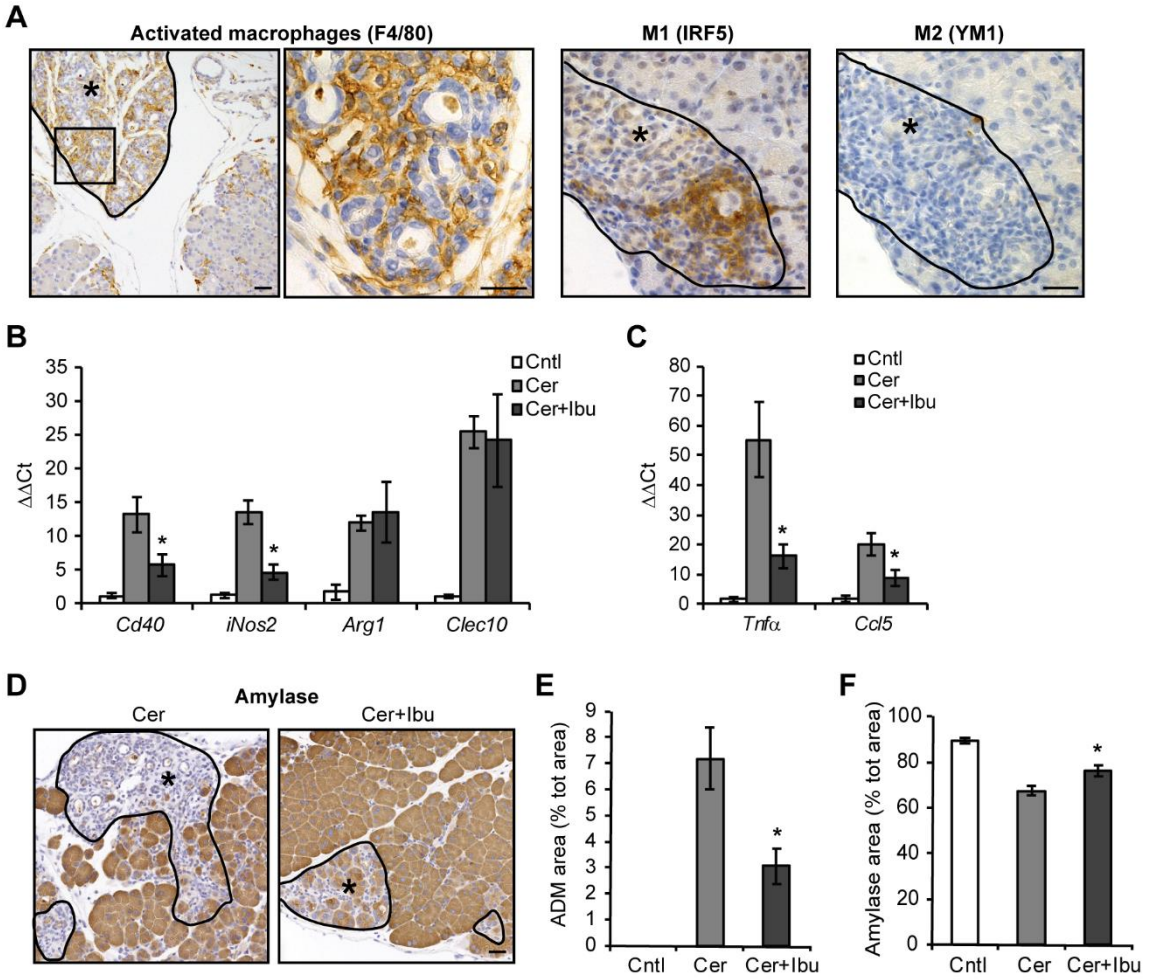

A
Activated macrophages (F4/80)
M1 (IRF5)
M2 (YM1)
B
ΔΔCt
Cntl
Cer
Cer+Ibu
Cd40
iNos2
Arg1
Clec10
C
ΔΔCt
Cntl
Cer
Cer+Ibu
Tnfα
Ccl5
D
Amylase
Cer
Cer+Ibu
E
ADM area (% tot area)
Cntl
Cer
Cer+Ibu
F
Amylase area (% tot area)
Cntl
Cer
Cer+Ibu

Figure 3

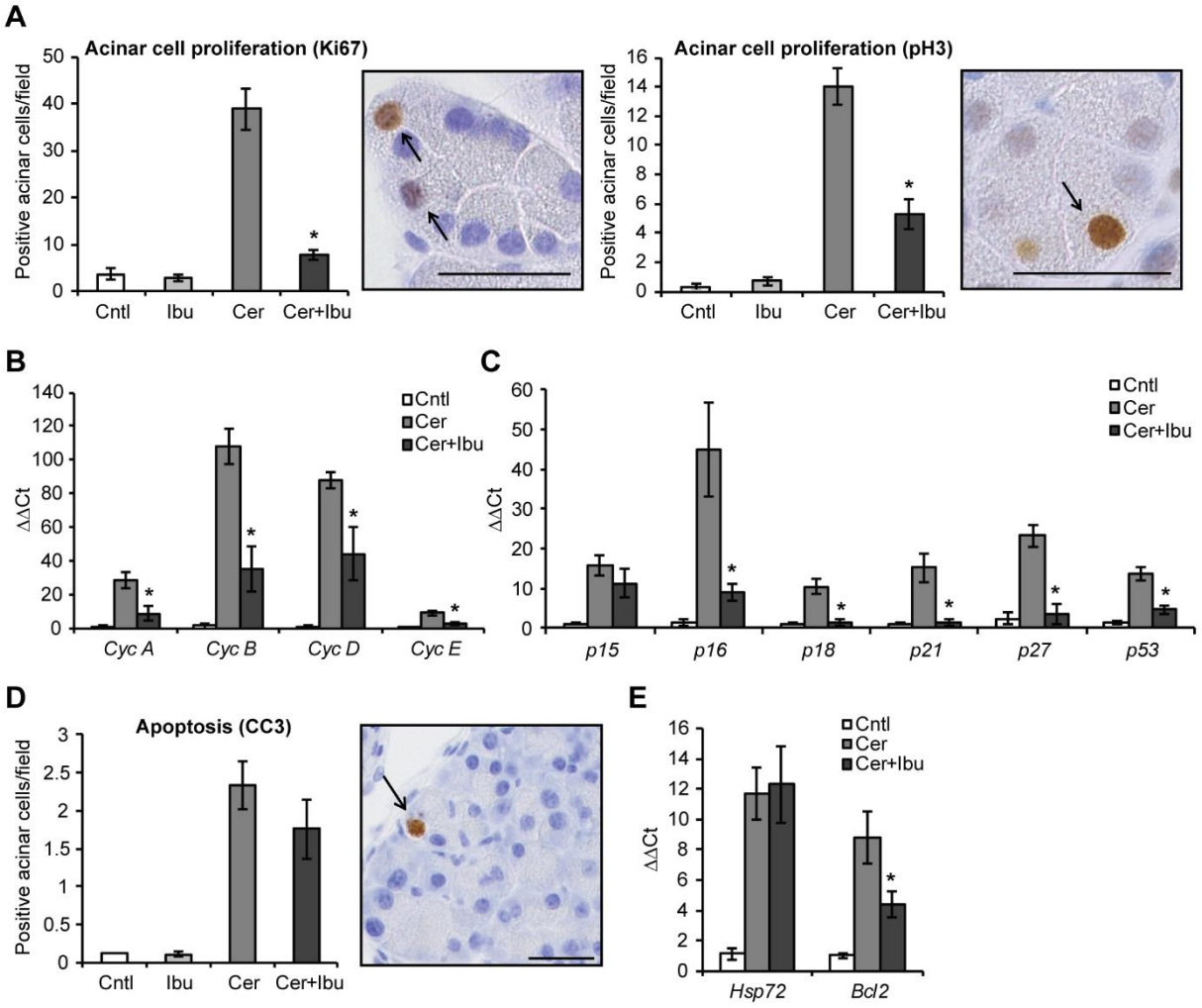

A
Acinar cell proliferation (Ki67)
Positive acinar cells/field
Cntl
Ibu
Cer
Cer+Ibu
Acinar cell proliferation (pH3)
B
ΔΔCt
Cyc A
Cyc B
Cyc D
Cyc E
C
p15
p16
p18
p21
p27
p53
D
Apoptosis (CC3)
E
Hsp72
Bcl2

Figure 4

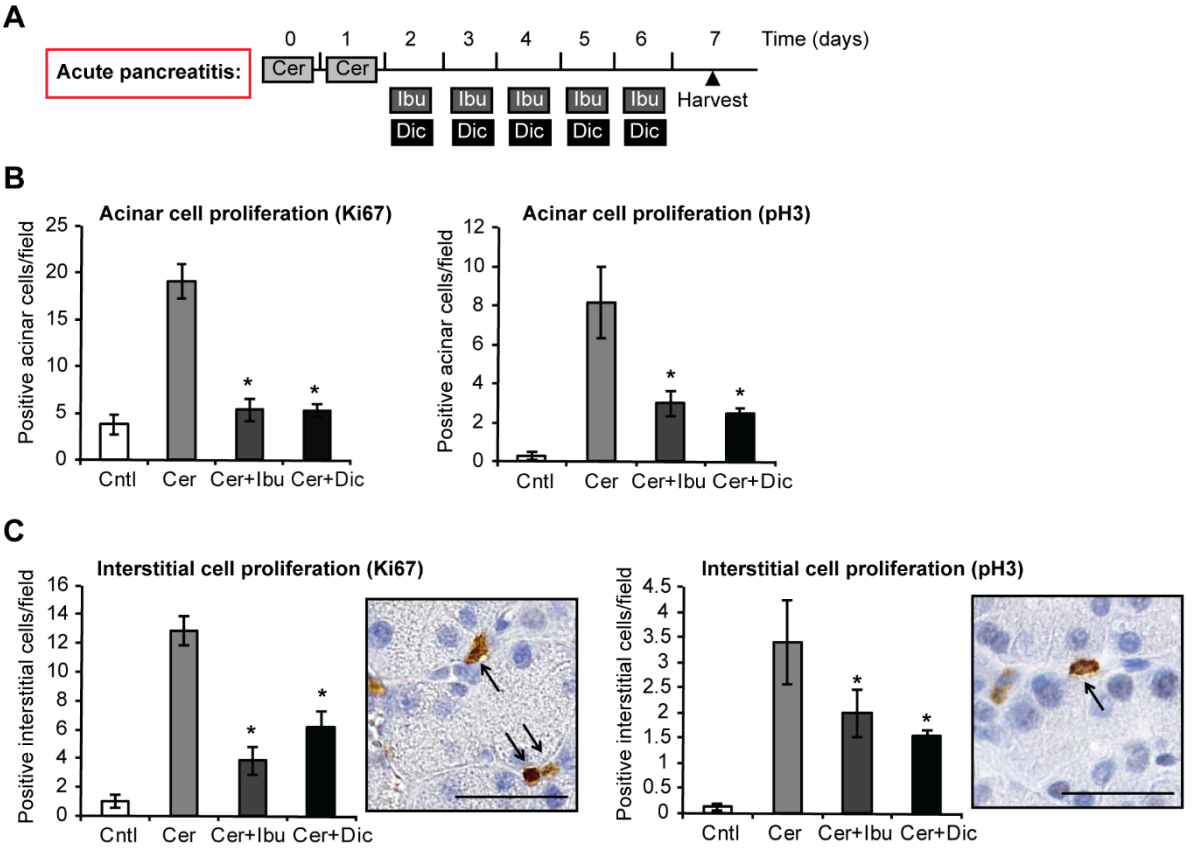

A
Acute pancreatitis:
0 1 2 3 4 5 6 7 Time (days)
Cer Cer
Ibu Ibu Ibu Ibu Ibu Harvest
Dic Dic Dic Dic Dic
B
Acinar cell proliferation (Ki67)
Positive acinar cells/field
25 20 15 10 5 0
Cntl Cer Cer+Ibu Cer+Dic
Acinar cell proliferation (pH3)
Positive acinar cells/field
12 10 8 6 4 2 0
Cntl Cer Cer+Ibu Cer+Dic
C
Interstitial cell proliferation (Ki67)
Positive interstitial cells/field
16 14 12 10 8 6 4 2 0
Cntl Cer Cer+Ibu Cer+Dic
Interstitial cell proliferation (pH3)
Positive interstitial cells/field
4.5 4 3.5 3 2.5 2 1.5 1 0.5 0
Cntl Cer Cer+Ibu Cer+Dic

Figure 5

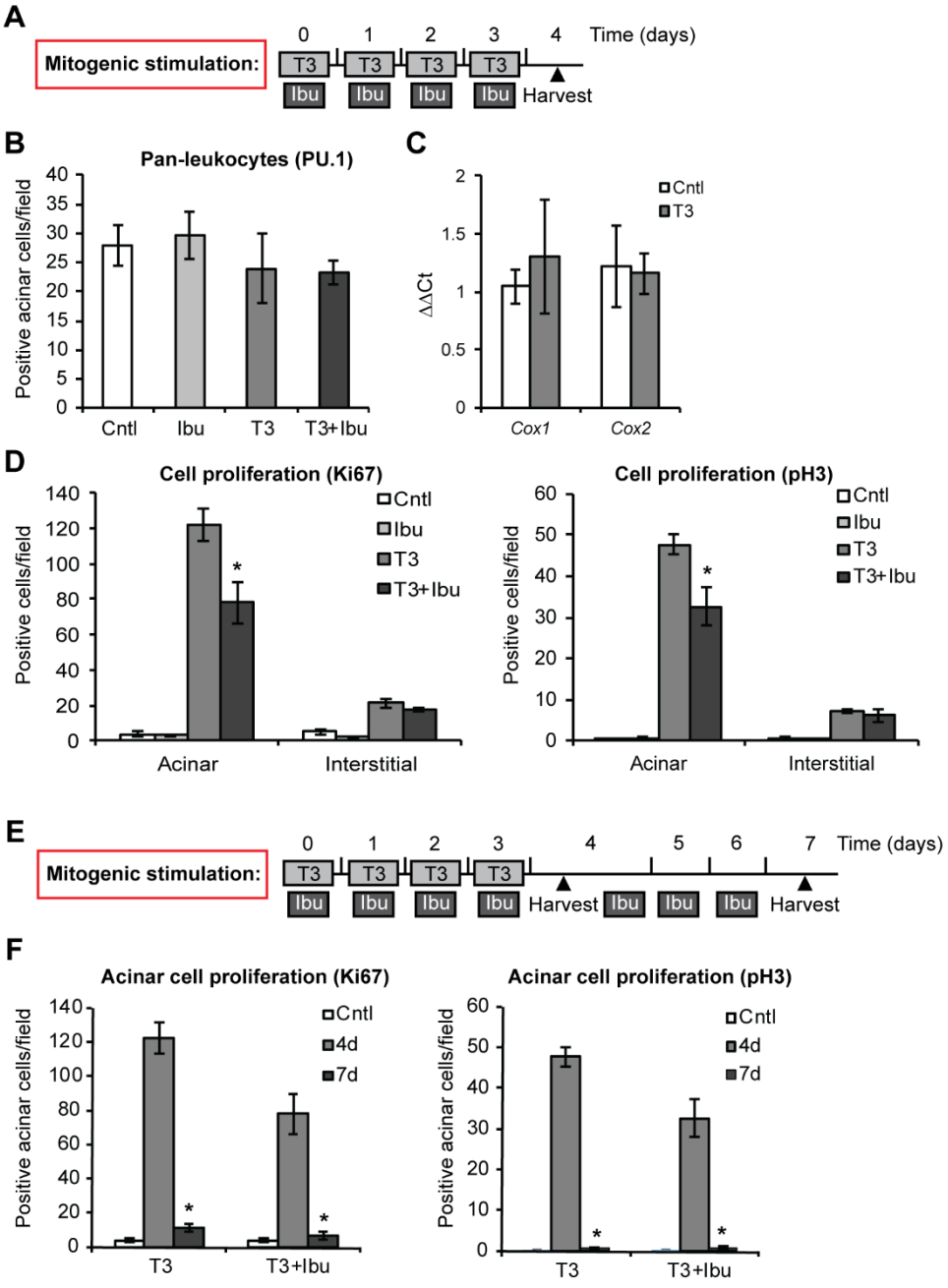

A
Mitogenic stimulation:
0 1 2 3 4 Time (days)
T3 T3 T3 T3
Ibu Ibu Ibu Ibu Harvest
B
Pan-leukocytes (PU.1)
Positive acinar cells/field
Cntl Ibu T3 T3+Ibu
C
ΔΔCt
Cntl
T3
Cox1 Cox2
D
Cell proliferation (Ki67)
Positive cells/field
Cntl
Ibu
T3
T3+Ibu
Acinar Interstitial
Cell proliferation (pH3)
E
Mitogenic stimulation:
0 1 2 3 4 5 6 7 Time (days)
Harvest Harvest
F
Acinar cell proliferation (Ki67)
Positive acinar cells/field
Cntl
4d
7d
T3 T3+Ibu
Acinar cell proliferation (pH3)

Figure 6

Supplementary figure legends

Supplementary figure 1. Quantification of weight loss, expressed as percentage of initial weight, in mice upon induction of acute pancreatitis. Results are average ± SEM (n=5), *P < 0.05.

Supplementary figure 2. qPCR of ADM marker Ck19. Results are average ± SEM (n=5), *P < 0.05.

Supplementary figure 3. (A) Quantification of TUNEL-positive apoptotic acinar cells after induction of pancreatitis. Right panel, representative microphotograph of stained cells. (B) Quantification of DNA damage in acinar cells upon γH2A.X staining after induction of pancreatitis. Right panel, representative microphotograph of stained cells. Results are average ± SEM (n=5), *P < 0.05. Scale bars: 50 µM.

Supplementary figure 4. Quantification of cleaved-caspase 3 (CC3)-positive apoptotic acinar cells following mitogenic stimulation with T3. Results are average ± SEM (n=5), *P < 0.05.

Supplementary figure 5. Quantification of live cell number after incubating AR42J acinar cells with different concentrations of ibuprofen for 72 hours. "Seeded" indicates the initial number of seeded cells. Results are average ± SEM (n=3), *P < 0.05.

Figure S1

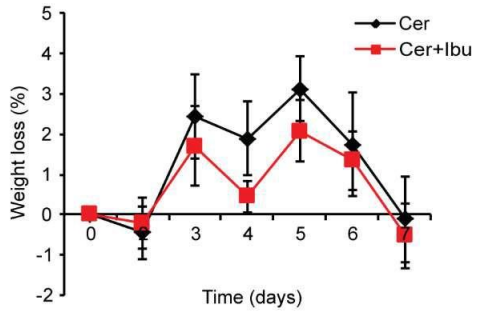


Figure S2

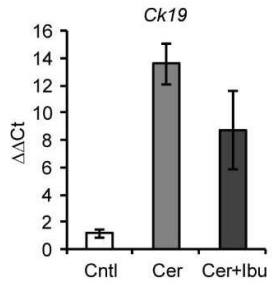


Figure S3

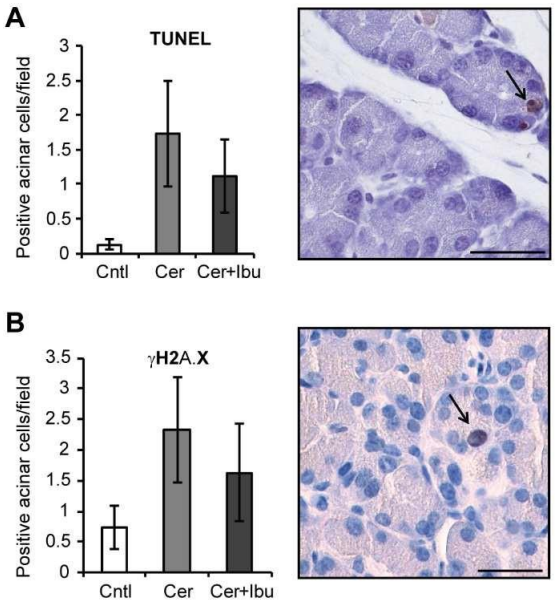


Figure S4

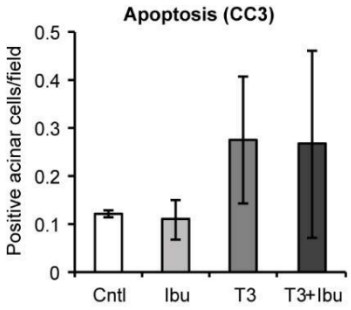
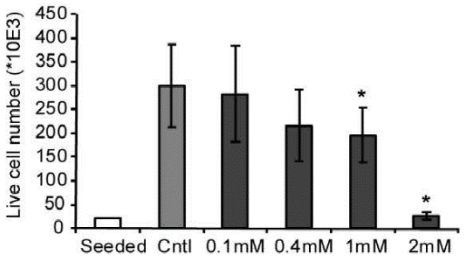


Figure S5



6. Discussion

Acute pancreatitis is a debilitating inflammation of the pancreas, which in its severe form is associated with substantial morbidity and mortality. Following pancreatitis, the pancreas has some regenerative capacities and differentiated acinar cells are able to re-enter the cell cycle and start to proliferate. However, the extent of pancreatic regeneration is limited; therefore elucidating the molecular mechanisms that drive pancreatic regeneration and boost this process is a major focus of our research. Thyroid hormones are pleiotropic factors involved in a variety of cellular processes that range from metabolism to regeneration. Here I will discuss our findings about the regulation and function of thyroid hormones during pancreatic regeneration (manuscript A). Furthermore, I will integrate in the discussion the results obtained using T3 as a mitogenic stimulus to study the mechanisms of acinar cell proliferation in the absence of inflammatory injury (Manuscript B, [166]).

6.1 Local regulation of TH signaling

One of the key aspects of TH signaling is the possibility to be activated in a cell specific fashion independently from systemic levels within the circulation. This regulation relies on the differential expression and activity of TH transporters and deiodinases enzymes, which define intracellular concentrations of TH. Many studies have highlighted that control of local TH availability constitutes an efficient mechanism to modulate cellular responses in the context of development, homeostasis and regeneration. In the present project, we sought to investigate whether acute pancreatitis alters local availability of TH in both a systemic and tissue specific manner. To this aim, we took advantage of the well establish model of serial cerulein injections to induce pancreatitis and quantified the levels of TH at different time points.

Our study design focuses on a one-week period that comprises the induction of damage to the recovery of pancreatic tissue, showing kinetics of disease development in line with previously reported observations. We can divide this process in the following phases, which in turn help us define and characterize pancreatic regeneration following acute pancreatitis (see figure 9):

1. **Induction of damage**: Cerulein treatment induces pancreatic damage by overstimulating secretion of digestive enzymes that are aberrantly released within the tissue and in the blood. Within the pancreas, premature activation of digestive enzymes leads to cell death and necrosis of the tissue. This process can be monitored by measuring the amount of digestive enzymes within the circulation as an indicator of the pancreatic damage.

2. **Inflammation of the pancreas:** consequent to the induction of damage, infiltration of inflammatory cells can be observed within the pancreatic tissue.

3. **Regeneration**: beginning about 48h from the first cerulein injections, a wave of acinar cell proliferation can be observed. Concomitantly, injured acinar cells may dedifferentiate and acquire a ductal phenotype (ADM).

4. **Recovery**: one week from the induction of pancreatitis, the pancreatic tissue appears normal with no signs of damage and inflammation; furthermore, proliferation returns to baseline values.

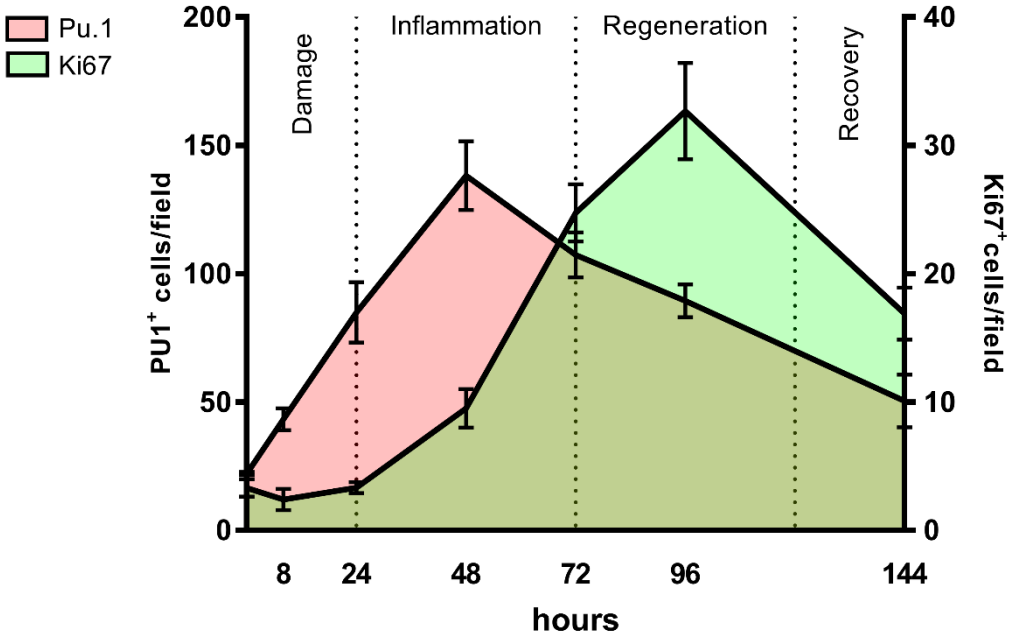


Figure 9*: Phases of acute pancreatitis. In the present graph the quantification of leucocytes infiltrating cells (Pu.1⁺ cells – left axis) and the number of proliferating acinar cells (Ki67⁺ - right axis) observed following cerulein treatment are presented. See above for a short description of the four phases proposed. To note, the following phases were chosen arbitrarily to help the discussion of the obtained results.*

We demonstrated that, following induction of damage, acinar cells are able to increase the levels of T3 within the pancreas. Importantly, this local hyperthyroidism precedes the beginning of the regenerative phase, offering a first hint on the putative involvement of TH in the regeneration of the pancreas. To validate and further investigate the observed regulation of TH, we characterized expression levels of deiodinases enzymes, which were in agreement with a local activation of TH.

Similarly to what we observed in the pancreas, TH are locally regulated in response to injury in various organs (Heart [167], Liver [168], Muscle [100, 101, 169]). Kester and coworkers for example, observed a regulation of deiodinases activities and TH levels in the liver following partial hepatectomy (a model of liver resection to study liver regeneration) [168], while Dentice and collaborators described the specific regulation of TH availability

within muscle progenitor cells (satellite cells) during skeletal muscle regeneration [100, 101, 169]. In this view, TH signaling may serve as a conserved regulator of tissue damage response. It should be restated that, due to the intricate network of biological processes regulated by TH, further studies are required to validate this hypothesis.

An interesting topic for future investigation is the identification of the factors that promote activation of TH signaling within the pancreas in response to acute pancreatitis. This is of particular interest for the characterization of the initial events that trigger pancreatic regeneration. Some of the factors known to promote DIO2 upregulation in multiple cellular contexts (i.e. Ca^{2+}/cAMP [65] , NFκB [170], Jnk [171]) have been reported to be upregulated at the onset of pancreatitis (Ca^{2+}/cAMP [172] , NFκB [173], Jnk [174]) and may therefore be involved in this process during pancreatic regeneration. Of note, the identification of the master regulators of pancreatic regeneration, which activate TH following acute pancreatitis, may provide novel means for the development of strategies to specifically modulate TH signaling within the pancreas.

Interestingly, in our model as in liver regeneration [168], induction of tissue injury leads to a decrease in circulating levels of TH. This pancreatitis-associated hypothyroidism has also been observed in humans [175]. More so, many reports have highlighted that patients hospitalized in intensive care units suffering from acute critical illnesses show decreased TH levels within the first hours [176, 177]. Furthermore, Abliz and coworkers observed that severe acute pancreatitis in rats induces inflammation of the thyroid, highlighting a direct association between pancreatic injury and thyroid status [178]. Although still poorly investigated, a possible explanation for this transient hypothyroidism may be ascribed to the increased consumption of TH within the injured tissue. The recognition of a direct link between pancreatitis and thyroid function is of interest because severe acute pancreatitis can lead to multi-organs dysfunction and hypothyroidism may be a contributing factor in the developing of those complications.

6.2 Effect of TH on pancreatic regeneration

Based on the observed regulation of TH signaling in response to acute pancreatitis, we decided to investigate the effect of TH during pancreatic regeneration. To this aim, we used established models of alteration of TH levels as well as GMO mice with pancreas specific increase of TH, and characterized pancreatic regeneration following acute pancreatitis.

We demonstrated a positive correlation between TH levels and the number of proliferating acinar cells, which provides an indication of the extent of pancreatic regenerative potential. These results are in agreement with previously reported observations that exogenous administration of T3 strongly stimulates acinar cell proliferation [86, 96, 165]. Moreover, supported by the characterization of the effect of systemic hypothyroidism

and peripheral inhibition of TH conversion, we proposed that local hyperthyroidism is an initiating event required for the induction of the regenerative phase following acute pancreatitis.

Interestingly, both in the liver [91] and in the pancreas, administration of T3 during the disease state induces more proliferation than observed in healthy conditions. This synergistic effect may account for external signaling promoting cell proliferation, as suggested in the liver [91], but may reflect also a more permissive cellular status towards increased TH activity.

No differences in the induction of damage were observed in any of the models used, demonstrating the absence of interference between alteration of TH levels and cerulein treatment. Although we did not characterize if changes in TH levels affect immunomodulatory properties of infiltrating cells, we did not observe any differences in the levels of inflammatory cell infiltration in any of the experimental groups, suggesting that TH do not alter the recruitment of infiltrating cells within the pancreatic tissue. This is of interest because previous studies highlighted that inflammatory cells are targets of TH activity [179, 180]; however, when TH function is studied in the context of regeneration, no changes in the inflammatory response were reported [82, 91, 93, 100, 101, 181]; therefore further investigation are needed to better characterize this process.

Apart from the actions of TH on immune cells, an additional topic for future investigations might be the role of inflammation in regulating TH signaling. Most of, if not all, the regenerative responses which show involvement of TH are in fact characterized by an inflammatory state of the tissue. In our model for example, recruitment of inflammatory cells partially overlaps with the increase in T3 concentration within the pancreatic tissue. Understanding whether inflammation is not a prerequisite for regeneration (defined as proliferation of acinar cells) may have important consequences in the development of novel therapies.

6.3 Molecular mechanisms of TH action

Taking into account the observed requirement of TH for pancreatic regeneration, we decided to investigate the molecular mechanisms of the T3-driven induction of acinar cell proliferation. To be able to focus solely on TH action, we supplemented T3 in the absence of an inflammatory reaction.

We found that T3-driven acinar cell proliferation involves activation of epigenetic modulators (HDACs) and the protein kinase B (PKB, also known as Akt), while TGF-β signaling acts as a negative regulator of TH induction of proliferation (Manuscript A). These targets were selected based on two selection criteria: 1) previous reports of interplay with TH signaling and 2) known activation within the pancreas following acute pancreatitis and involvement in acinar cell proliferation. Interestingly, we observed that inhibition of each of the investigated pathways affects acinar cell proliferation similarly in the disease state or upon T3 stimulation. This in turn suggests that exogenous T3 administration mimics acinar cell proliferation induced by pancreatitis.

TH regulate many responses in a cell specific manner, therefore comparison of TH role in the regeneration of different organs is difficult. Nevertheless, we can try to highlight similarities while comparing organs that respond to TH. As previously mentioned in the introduction of this thesis, examples of organs in which TH stimulate proliferation are liver, skin, and to a certain extend the heart [81, 86, 182]. The Pi3k/Akt signaling cascade is amongst studied signaling in regenerative processes and regulates proliferation in many tissues; its crucial role in liver regeneration has been demonstrated [183] but evidences that TH can activate this signaling in hepatocytes have not yet been provided. Analogously, Pi3k/Akt signaling was demonstrated to participate in skin and heart regeneration [184, 185]; with regards to the skin, indirect indications that TH may modulate this pathway in keratinocytes come from the analysis of GMO lacking both TH receptors, which showed different levels of phosphorylated-Akt compared to Wt [80]. In the heart, Kenessey and Ojamaa demonstrated that T3 activates the Pi3k/Akt cascade in a TH receptor α1 dependent mechanism, while Mourouzis and coworkers highlighted that T3 stimulation of Akt phosphorylation is dose dependent [186, 187].

The TGF-β signaling, which we demonstrated to act as a restrictor of acinar cell proliferation following acute pancreatitis [151] and upon mitogenic stimulation (Manuscript A), is a well known regulator of pancreatic regeneration. Particularly, its function is often studied when damage persists and tissue fibrosis occurs. In line with our observations, the antagonism between TH and TGF-β signaling has been observed at multiple levels in various tissues: for example, TH receptor β is able to inhibit SMAD dependent transcription (a downstream effector of TGF-β), reducing liver fibrosis [188], while TGF-β blocks TH action by promoting expression of deiodinase type-III in lung fibroblasts and other cells [189]. Interestingly, TGF-β can impair muscle regeneration by inhibiting differentiation of myoblasts [190]. It is tempting to speculate that this effect of TGF-β relies on the persistent expression of deiodinase type-III. As previously mentioned, deiodinase type-III is upregulated in skeletal muscle progenitors while they proliferate and its repression is required to promote TH-driven myofibers differentiation [100, 101]. In addition, Safer and colleagues observed divergent effects of TH on skin proliferation based on the route of administration. Briefly, while topical application of T3 induced keratinocytes proliferation, systemic administration of T3 (injected intraperitoneal) reduced dermal thickening and proliferation. The authors proposed that the reduction in proliferating keratinocytes, observed when T3 was administered systemically, could be attributed to increased production of TGF-β by the dermal fibroblasts [81].

It is important to mention that previously, Kowalik took advantage of a selective agonist for the TH receptor β isoform (GC-01) and highlighted that T3-induced mitogenic stimulation of pancreatic acinar cells (and liver hepatocytes) is dependent on TH receptor β [96]. In our analysis we did not address the relative contribution of TH receptors or other partners, therefore the results presented should be seen as a “generic” TH response.

Overall, further analyses are required to better understand the similarities or the differences between TH modulation of these pathways in different tissues.

6.4 The use of TH to study acinar cell proliferation *in vivo*

While endogenous T3 is required for pancreatic regeneration following pancreatitis, its exogenous administration to healthy mice offers a unique model to study acinar cell proliferation *in vivo*. In the following paragraph I will discuss the potential use of this model starting from examples already performed by our lab. Pancreatic regeneration includes damage, inflammation, and the participation of multiple cells populations. Considering the intricate network of interactions within and amongst the cells, the possibility to discriminate the contribution of a selected target to a specific trigger (i.e. damage, inflammation etc.) remains difficult. In this view, TH administration may constitute an efficient model to dissect the relative contribution of a selected target with regards to acinar cell proliferation without considering damage or inflammation. Indeed, our data demonstrated that TH administration, both in healthy condition and during pancreatitis, promotes acinar cell proliferation *in vivo* without inducing damage or inflammation.

Our lab took advantage of the mitogenic properties of TH to test the effect of selected targets on acinar cell proliferation. In manuscript B for example, we investigated the effect of a nonsteroidal anti-inflammatory drug (NSAID), Ibuprofen, on pancreatic regeneration following acute pancreatitis. Ibuprofen is commonly administered to patients suffering from mild cases of acute pancreatitis to relieve abdominal pain. To mimic this situation, we administered Ibuprofen to mice in a therapeutic regimen following induction of pancreatitis, and we analyzed putative changes in the inflammatory and the regenerative responses. We found that Ibuprofen treatment reduced both the level of inflammation and the number of proliferating acinar cells. To test if the reduction of acinar cell proliferation was a consequence of reduced inflammation, we stimulated acinar proliferation with T3 with or without Ibuprofen. Our results demonstrated that Ibuprofen reduces proliferation independently of inflammation. The observation that Ibuprofen directly affects pancreatic acinar cell proliferation is of clinical interest because it highlights an undesirable side effect. This in turn provides an example of putative implementation for the “T3-model” to test potential side effects of therapeutic compounds with regards to acinar cell proliferation.

Similarly, we adopted an analogous approach when studying the effect of Serotonin in pancreatic regeneration following pancreatitis [166]. Briefly, we wanted to characterize if absence of peripheral Serotonin (mice lacking of tryptophan hydroxylase 1, TPH-KO) altered acinar cell proliferation; to this aim we administered T3 to WT and KO mice and compared the number of proliferating acini. Thanks to this model, we could demonstrate that absence of peripheral Serotonin does not influence acinar cell proliferation upon T3 stimulation. More so, we exploited the “T3-model” to also investigate differences in acinar cell proliferation between mouse strains. Particularly, we compared the MRL/MpJ mouse strain to the common B6 response to pancreatitis (paper under revision). We observed that following induction of acute pancreatitis, MRL mice had a greater inflammatory reaction and higher number of proliferating acinar cells. However, when compared by T3 stimulation, both strains showed comparable degree of proliferation; in turn, thanks to the use of T3 we could demonstrate that

other factors, and not acinar cells per se, are responsible for the higher regenerative response observed in the MRL strain.

6.5 Limitation to the study

The present project aimed to characterize the putative involvement of TH in an experimental model of pancreatic regeneration. In this view, the major limitation of the study is possibly the absence of a reporter-based system for tracking thyroid hormone status at a cellular level. Indeed the presence of a reporter would have allowed gaining more insight on many processes, such as:

1. Investigate if local hyperthyroidism is a shared event among acinar cells in response to pancreatitis or only selected cells modulate TH levels
2. Confirm that hyperthyroid acinar cells proliferate
3. Investigate the TH status of other pancreatic cells (i.e. ductal or endocrine cells)

Importantly for future research, a TRE-luciferase knock-IN mouse model has recently been generated [191]. This model will allow monitoring TH receptors transcriptional activity and test genomic action of T3 *in vivo.* Additionally, in the present project we focused our analysis on pathways known to be potentially regulated by T3, in this regard the use of –omics approaches may unveil novel targets of TH within the pancreatic tissue.

Furthermore, here we characterized the effect of acinar specific deletion of deiodinase type-III, the main inactivator of TH. Therefore, future studies should focus on the role of type-I and type-II deiodinase in pancreatic regeneration. The generation of an acinar-specific KO model for the two deiodinases will in turn provide a tool to study the relative contribution of each enzyme in generating a transient local hypothyroidism at the onset of acute pancreatitis.

Lastly, future studies are needed to investigate the importance of TH in other models of pancreatic damage, such as pancreatectomy and chronic pancreatitis.

7. Conclusive remarks

Our findings highlight an important role of thyroid hormones in promoting acinar cell proliferation following acute pancreatitis. Many aspects of TH function within the pancreas remain elusive and offer inspiration for further studies. An intriguing observation that emerged is the striking similarity between pancreatic and liver with regards to TH mitogenic activity and the dynamics of regeneration. It would be interesting to investigate whether a transient local hyperthyroidism occurs during liver regeneration; however considering the high expression of deiodinase type I within the liver the mechanisms beyond may be remarkably different. Furthermore, our study provide the foundation for the development of novel therapies that aim to boost exocrine regeneration. Finally, we propose the use of T3-mitogenic activity for the study of the effect of potential therapeutics on acinar cell proliferation.

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Curriculum vitae

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Education

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PhD Thesis title: Role of thyroid hormones in pancreatic acinar cell regeneration following acute pancreatitis

Supervisors: Prof. Rolf Graf, PD Dr. Sabrina Sonda

2011 – 2013: M.A. in Molecular and Cell biology at University of Pisa, Italy.

Master Thesis title: Isolation and characterization of Mesenchymal stromal cells from the canine liver

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2008 - 2011: B.A. In Molecular and Cell biology at University of Pisa, Italy.

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Supervisors: Prof. Luciana Dente, Dr. Silvia Marracci

2003 - 2008: Liceo scientifico E. Majorana, Latina (LT), Italy. Maturity certificate.

Additional training

2017: From the transcriptome to the proteome – a practical course. FGCZ, Zürich, Switzerland.

2016: Course in Network and Pathways analysis. EMBL-EBI, Wellcome Genome Campus, Hinxton, Cambridge UK.

2015: Course in Next generation sequencing. FGCZ, Zürich, Switzerland.

2015: Flow cytometry course. University of Zürich, Switzerland.

2015: Course in laboratory animal science (LTK Module I). FELASA, Zürich, Switzerland.

2015: Erasmus placement project. University of Utrecht, Department of companion animal life sciences, The Netherlands.

Awards

2016: Best poster award at imMED retreat 2016, 13-06-2016, (ZIHP) Magglingen

Publications

2018: Enhanced proliferation of pancreatic acinar cells in MRL/MpJ mice is driven by severe acinar injury but independent of inflammation. Bombardo M., Malagola E., Chen R., Carta A., Seleznik GM., Hills AP., Graf R., Sonda S. **Sci Rep.** 2018 Jun 20;8(1):9391. doi: 10.1038/s41598-018-27422-0.

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